Universidade de Lisboa

Faculdade de Farmácia

EVALUATION OF NEUROPROTECTIVE AND NEUROTOXIC FUNCTIONS OF DIFFERENT MICROGLIA PHENOTYPES IN ALZHEIMER'S DISEASE ONSET AND PROGRESSION, USING *IN VITRO* AND *IN VIVO* MODELS

Cláudia Alexandra Oliveira Lopes Caldeira

Orientador(es): Prof. Doutora Dora Maria Tuna de Oliveira Brites
Prof. Doutora Adelaide Maria Afonso Fernandes Borralho

Tese especialmente elaborada para obtenção do grau de Doutor em Farmácia, Biologia Celular e Molecular

2017

Universidade de Lisboa
Faculdade de Farmácia

EVALUATION OF NEUROPROTECTIVE AND NEUROTOXIC FUNCTIONS OF DIFFERENT MICROGLIA PHENOTYPES IN ALZHEIMER'S DISEASE ONSET AND PROGRESSION, USING *IN VITRO* AND *IN VIVO* MODELS

Cláudia Alexandra Oliveira Lopes Caldeira

Orientador(es): Prof. Doutora Dora Maria Tuna de Oliveira Brites
Prof. Doutora Adelaide Maria Afonso Fernandes Borralho

Tese especialmente elaborada para obtenção do grau de Doutor em Farmácia, Biologia Celular e Molecular

Júri:

Presidente: Doutora Matilde da Luz dos Santos Duque da Fonseca e Castro, Professora Catedrática e Diretora da Faculdade de Farmácia da Universidade de Lisboa, Presidente do júri por delegação de competências;

Vogais:

- Doutora Cláudia Margarida Gonçalves Cavadas, Professora Auxiliar
  Faculdade de Farmácia da Universidade de Coimbra;
- Doutora Paula Isabel da Silva Moreira, Professora Auxiliar
  Faculdade de Medicina da Universidade de Coimbra;
- Doutora Fernanda Cristina Gomes de Sousa Marques, Investigadora Auxiliar
  Escola de Medicina da Universidade do Minho;
- Doutor Alexandre Valério de Mendonça, Investigador Coordenador
  Faculdade de Medicina da Universidade de Lisboa;
- Doutora Dora Maria Tuna de Oliveira Brites, Investigadora Coordenadora
  Faculdade de Farmácia da Universidade de Lisboa, Orientadora.

2017

Para a elaboração da presente tese de doutoramento foram usados integralmente como capítulos, artigos científicos já publicados ou submetidos para publicação, em revistas científicas internacionais indexadas. Estes trabalhos desenvolvidos foram realizados sob a orientação científica de Dora Brites e Adelaide Fernandes e neles participaram: Ana Filipa Oliveira, Ana Rita Vaz, Ana Sofia Falcão, Andreia Barateiro, Carolina Cunha, Elisabete Ferreiro, Elsa Seixas, Adelaide Fernandes e Dora Brites.

De acordo com o disposto no ponto 1 do artigo nº41 do Regulamento de Estudos Pós-Graduados da Universidade de Lisboa, deliberação nº 93/2006, publicada em Diário da República – II Série nº 153 – 5 de Julho de 2003, a Autora desta dissertação declara ter sido a principal executante do trabalho experimental, tendo ativamente participado na conceção do desenho experimental, na interpretação dos resultados obtidos e na redação dos manuscritos para publicação.

Os estudos apresentados nesta dissertação foram realizados no grupo de investigação "Neuron Glia Biology in Health & Disease", do Research Institute of Medicines (iMed.ULisboa), da Faculdade de Farmácia da Universidade de Lisboa, sob a orientação das Professoras Doutoras Dora Brites e Adelaide Fernandes.

O trabalho foi subsidiado por FEDER (COMPETE Programme), por fundos Nacionais (FCT – Fundação para a Ciência e a Tecnologia) Projeto FCT-PEst-OE/SAU/UI4013 no âmbito do Financiamento Plurianual ao iMed.ULisboa, Projeto PTDC/SAU-FAR/118787/2010 da responsabilidade da Professora Doutora Dora Brites, Projecto FCT-EXPL/NEU-NMC/1003/2013 da responsabilidade da Professora Doutora Adelaide Fernandes, e, em parte, por EU Joint Programme – Neurodegenerative Disease Research (JPND) MADGIC Projeto JPco-ND/0003/2015 da responsabilidade da Professora Doutora Dora Brites. Foi ainda subsidiado pelo prémio Edgar Cruz e Silva 2012 do Grupo de Estudo do Envelhecimento Cerebral e Demência concedido à Professora Doutora Dora Brites, e pela bolsa da Universidade de Lisboa/Fundação Amadeu Dias concedida à Professora Doutora Adelaide Fernandes.

Para os meus filhos, marido e família

Acknowledgements/Agradecimentos

É de todo impossível expressar em palavras o agradecimento que tenho a todas as pessoas, que de uma maneira ou de outra, me ajudaram a concluir esta etapa da minha vida. Espero não me esquecer de ninguém… mas, se isso acontecer, no meu coração jamais serão esquecidos.

Em primeiro lugar gostaria de agradecer às minhas orientadoras, Professora Doutora Dora Brites e Professora Doutora Adelaide Fernandes.

À Professora Dora gostaria de agradecer por me ter acolhido no seu grupo. Agradeço por ter sido uma orientadora sempre presente, pelos seus inúmeros conselhos, por tudo o que me ensinou, por toda a ajuda, e também pela paciência que demonstrou ao longo destes anos com esta "Engenheira Agrónoma". Obrigada por ter acreditado e confiado em mim. Obrigada por tudo!

À Adelaide, para quem não tenho palavras de agradecimento… Além de minha coorientadora, foste uma grande AMIGA com quem pude contar nos bons, e principalmente nos maus momentos. E ambas sabemos, que ao longo destes anos, houve uns quantos…☺ A nossa amizade é muito importante para mim, e tive muita sorte em te ter como minha coorientadora! Obrigada por toda a ajuda, paciência, disponibilidade e amizade. Estavas sempre lá! Sem ti não teria conseguido ☺. Sempre que precisares de mim, estarei ao dispor…é só apitar! OBRIGADA do fundo do meu coração!

Gostaria também de agradecer aos restantes elementos do grupo Neuron Glia Biology in Health and Disease. Ao Professor Rui agradeço a sua boa disposição durante o almoço e na hora do cafezinho, a amizade, além das preciosas e importantes dicas. Obrigada! À Professora Alexandra agradeço todos os conselhos e dicas que me deu, sempre com um sorriso no rosto, além da amizade e de todo o apoio. Muito obrigada!

Agradeço também às "meninas" do gabinete pela ENORME ajuda e amizade que sempre me deram. Vocês foram pilares muito importantes nesta minha etapa! Ritinha…como sabes, não tenho forma de agradecer em palavras toda a tua ajuda e grande amizade. O meu primeiro contacto com as células animais foi contigo… Eras a "mamã" e eu e a Carol os teus "patinhos"… Agradeço-te do fundo do coração a muita ajuda, disponibilidade e paciência, que sempre tiveste comigo. Mas houve também muitas horas de boa conversa e muito riso (…sempre fora do horário laboratorial, claro!!… ☺). Cresceu uma grande amizade, que espero que continue…e podes sempre contar comigo…Tu sabes que sim! OBRIGADA! Sofia, não me vou esquecer do teu

"chop-chop?" a chamar o pessoal para o almocinho… A tua companhia no almoço tornava tudo mais alegre. Muito te agradeço as boas conversas de "boleia" até à Amadora, na fila do IC19. Vou ter saudades ☺. Agradeço-te também toda a ajuda e disponibilidade que sempre tiveste comigo. Foste muito importante! A nossa amizade cresceu e sabes que também podes contar sempre comigo "para o que der e vier". OBRIGADA! Andreia, o teu sorriso é espectacular e a tua ternura maravilhosa. És um doce de pessoa! Agradeço-te toda a amizade, boa disposição e boas conversas que sempre fomos tendo. Obrigada por toda a tua ajuda e disponibilidade…Se precisares de mim, cá estarei! OBRIGADA!

Às meninas mais novas, obrigada por serem quem são! A vossa energia e alegria são contagiantes! Carolina (Carol), tu enches a cave de "sol"… e bem é preciso ☺. O facto de seres do signo Carneiro e com raízes de Ceira só podiam dar nisso ☺. Os nossos primeiros "passos" no laboratório foram em conjunto e, para mim, foram maravilhosos! Uma musiquinha brasileira no fluxo era o quanto bastava para esquecer o calor que se fazia sentir ☺. Adorei a ida a Tomar, e principalmente a Glia de Berlim. Foste uma filha "emprestada" que os meus miúdos adoraram! Muito te agradeço, além da amizade, toda a ajuda, disponibilidade e paciência que sempre tiveste comigo. És uma pessoa fantástica e eu gosto muito de ti! OBRIGADA! Cátia (Caty), a tua doçura e delicadeza são maravilhosas! És uma querida e a tua amizade estará sempre no meu coração. Muito te agradeço toda a disponibilidade e paciência que tiveste sempre para me ajudar. Foste incansável! OBRIGADA! Gisela (Gi), adorei as nossas conversinhas… És uma fofa e uma querida! Agradeço muito toda a ajuda, disponibilidade e amizade que sempre me deste. O teu apoio foi muito importante para mim! OBRIGADA! Muito boa sorte para vocês as três, do fundo do coração!!!! Marta, obrigada pela tua amizade e boa disposição, além de disponibilidade para me ajudar. És impecável! Boa sorte para ti, também! Adoro-vos a todas!!!

A todos os meninos e meninas de mestrado que de uma maneira ou de outra me ajudaram e contribuíram para momentos muito bem passados: Sara, Pedro, Catarina, Ana Rita, Carla, Maria, Mafalda, Rui, Filipe, Vera, Maria Inês, Catarina, Andreia, Cátia J e o grande Gonçalo. Também a doutoranda Inês F não pode ser esquecida. Obrigada a todos!

Não me posso esquecer das agora doutoras Filipa e Inês, que me acompanharam e ajudaram ao longo das suas teses de doutoramento. Obrigada pela amizade e boas conversas! Não me esquecerei das nossas agradáveis conversinhas matinais, Filipa ☺.

Ao André Frederico, Joana Rodrigues e Joana Serrano agradeço toda a ajuda e suporte que me deram. Obrigada pessoal!

A todas as pessoas do CPM um grande muito obrigada! Em particular ao grupo da Professora Cecília com quem partilhei algumas dúvidas e questões, e também amizade: Ana Luísa, Sofia, Marta, Hugo, Diane, Andreia, André(s), Maria(s), Pedro(s), Rui, Susana, Joana, …. E aos ex-grupo Duarte e Pedro Borralho, que muitas saudades deixaram. Miguel, muito obrigada por toda a tua amizade. Cuida bem da princesa Caty! Não há muitas como ela ☺

Ao Hugo (Botelho), que muito me surpreendeu com os seus dotes para a dança ☺, um obrigada especial por toda a amizade e simpatia que sempre demonstrou comigo. E um grande obrigada pelo suporte técnico, que muito jeito nos deu ☺

À minha família, o pilar da minha força, não tenho palavras, nem beijos, nem abraços que possam agradecer a cada um de vós o apoio e confiança que sempre me deram.

Aos meus queridos Filhos Rodrigo e Ricardo, e Marido Renato agradeço todo o apoio e conforto que me deram. Peço desculpa pelos momentos de mau humor, ou a falta de paciência que por vezes me atingiram. Sabem que vos adoro e que estão e estarão sempre em primeiro lugar em qualquer coisa que faça na vida. Vocês são a minha prioridade! Meu amor, estes quase 27 anos de convivência têm sido maravilhosos! Os altos e baixos da vida só nos tornam mais fortes e unidos! OBRIGADA por tudo e por seres quem és! AMO-TE MUITO! Meus filhos, o vosso sorriso é uma dádiva que muito me ajuda e alegra. Os vossos abraços, “mariquices” e beijinhos aquecem-me a alma e o coração. Não sei como seria a minha vida sem vocês! A mãe AMA-VOS MUITO! Nunca se esqueçam disso e nunca ponham isso em causa! OBRIGADAAAAA!!!

Aos meus Pais, o pilar da minha criação e formação, agradeço todo o carinho, ajuda e confiança que sempre depositaram em mim. Sem vocês não seria quem sou e não teria chegado a onde cheguei. OBRIGADA por terem sempre acreditado em mim e por me amarem tanto. Posso não dizer muitas vezes, mas também vos AMO MUITO!

Ao meu Irmão Miguel, cunhada Vânia e sobrinha/afilhada fofucha Bárbara (Bá), agradeço todo o apoio e carinho que sempre me deram. Vocês sabem a importância que têm na minha vida! Sei que posso sempre contar com vocês. A tua agitação dá-me muita alegria, fofucha da tia ☺ OBRIGADA! Também vos AMO e ADORO MUITO! Serás sempre o meu maninho Migó!

Aos meus Sogros Manelinha e Tito, agradeço toda a ajuda e apoio que sempre me deram. Foram muito importantes nesta minha conquista! Passámos por tempos muito

difíceis, mas que têm sido ultrapassados e assim continuará, tudo “jóia”!!!. Força!! OBRIGADA por tudo! Também vos ADORO!

À Avó Manela que sempre me apoiou, muito OBRIGADA! A todos os avós que já partiram, e que são estrelinhas lá no céu aos olhos dos meus filhos, OBRIGADA por sempre terem acreditado em mim. Padrinho, Ângelo, Henrique, Lourdes, Mané e Lelé continuem a olhar por mim! À minha querida Avó Madrinha, o meu Anjo da Guarda que partiu tão bruscamente, que sempre acreditou em mim, que sempre me apoiou e que tão bem me queria. Sem saber ler nem escrever, sempre compreendeu o meu esforço. Tínhamos longas e boas conversas ao telefone, com grandes gargalhadas, das quais tenho muitas saudades. Havia coisas que só nós as duas entendíamos… Sei que vai estar lá no alto sempre a olhar por mim, e eu passo longos períodos a olhar para o céu a falar consigo, minha querida Avó. OBRIGADAAAAA!

A todos os outros familiares e amigos que de qualquer forma me ajudaram a ultrapassar todos os obstáculos e dificuldades, um OBRIGADA muito grande!

MUITO OBRIGADA!

Index

Figure Index

CHAPTER 1 – GENERAL INTRODUCTION

CHAPTER 2 – MICROGLIA CHANGE FROM A REACTIVE TO AN AGE-LIKE PHENOTYPE WITH THE TIME IN CULTURE

CHAPTER 3 – KEY AGEING-ASSOCIATED ALTERATIONS IN PRIMARY MICROGLIA RESPONSE TO BETA-AMYLOID STIMULATION

CHAPTER 4 – REDUCED MICROGLIAL RESPONSIVENESS IN THE EARLY ALZHEIMER'S DISEASE STAGE OF 3xTg-AD MICE PRECEDE INFLAMMATION

CHAPTER 5 – FINAL CONSIDERATIONS

Table Index

Abbreviations

AD	Alzheimer's disease
Aβ	Amyloid-β
Aβo	Amyloid-β oligomer
Aβf	Amyloid-β fibrillary
AChEI	Acetylcholinesterase inhibitor
ADP	Adenosine diphosphate
AICD	Amyloid precursor protein intracellular domain
AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazolepropionic acid
APC	Antigen presenting cells
ApoE	Apolipoprotein E
APP	Amyloid precursor protein
ATP	Adenosine tri-phosphate
ATX	Autotaxin
BACE1	β-site amyloid precursor protein-cleaving enzyme 1
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
CCL	Chemokine motif ligand
CCR	Chemokine receptor
CD	Cluster of differentiation
CEBP	CCAAT/enhancer-binding protein
CNS	Central nervous system
CR	Complement receptor
CSF	Cerebrospinal fluid
CX3CL1	Chemokine (C-X3-C-motif) ligand 1 or Fractalkine
CX3CR1	Chemokine (C-X3-C-motif) receptor 1
Cyt *c*	Cytochrome *c*
DAMP	Danger-associated molecular pattern
DIV	Days *in vitro*
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Desoxyribonucleic acid
EAA	Excitatory amino acid
EDTA	Ethylenediamine tetraacetic acid
FAD	Early-onset Alzheimer's disease
FBS	Fetal bovine serum

FDA	Food and drug administration
FDG	Fluorodeoxyglucose
FIZZ1	Found in inflammatory zone 1
GFAP	Glial fibrillary acidic protein
GM-CSF	Granulocyte/macrophage-colony stimulating factor
GTL	Glutamate transporter
HMGB1	High-mobility group box 1
Iba1	Ionized calcium-binding adapter molecule 1
IDE	Insulin-degrading enzyme
IFN	Interferon
IGF	Insulin-like growth factor
IL	Interleukin
IL-1ra	Interleukin 1 receptor antagonist
iNOS	Inducible nitric oxide synthase
iPCS	Induced-pluripotent stem cell
LC3	Microtubule-associated-protein-light-chain-3
LOAD	Late-onset Alzheimer's disease
LPS	Lipopolysaccharide
MAP	Microtubule associated protein
MCI	Mild cognitive impairment
MCP	Monocyte chemotactic protein
MFG-E8	Milk factor globule-8
mGluR	Metabotropic glutamate receptor
MHC class II	Major histocompatibility complex class II
MIP	Macrophage inflammatory protein
miRNA	Micro ribonucleic acid
MMP	Matrix metalloproteinase
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
mtDNA	Mitochondrial desoxyribonucleic acid
NEP	Neprilysin
NF-κB	Nuclear factor-kappaB
NFT	Neurofibrillary tangle
NFTA	Nuclear factor of activated T cells
NGF	Neural growth factor
NLR	Nucleotide-binding oligomerization domain-like receptor

NLRP3	NOD-like receptor family pyrin domain containing 3
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain
NRG	Neuregulin
NSAID	Nonsteroidal anti-inflammatory drug
OPC	Oligodendrocyte precursor cells
PAMP	Pathogen-associated molecular patterns
PBS	Phosphate-buffer saline
PCR	Polymerase chain reaction
PET	Positron emission tomography
PRR	Pattern recognition receptor
PS	Presenilin
Ps	Phosphatidylserine
PsR	Phosphatidylserine receptor
PVP	Polyvinylpyrrolidone
qRT-PCR	Quantitative Real time-polymerase chain reaction
RAGE	Receptor for advanced glycation endproducts
RNS	Reactive nitrogen species
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Real time-polymerase chain reaction
SA-β-gal	Senescence-associated β-galactosidase
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SIRPα	Signal-regulatory protein α receptor
SOCS	Suppressor of cytokine signalling
SOD	Superoxide dismutase
SR	Scavenger receptor
Tau	Tubulin-associated unit
TGF	Transforming growth factor
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TREM	Triggering receptor expressed on myeloid cells
UTR	Untranslated region
UV	Ultra violet

VR	Vibronectin receptor
WT	Wild type
Ym1	Chitinase 3-like protein 3
α7nAchR	α7 nicotinic acetylcholine receptor
3xTg-AD	Triple transgenic mice model of Alzheimer's disease

Publications and Communications

Papers in international journals

1. **Caldeira C**, Cunha C, Vaz AR, Ferreiro E, Fernandes A, Brites D. Reduced microglial responsiveness in the early Alzheimer's disease stage of 3xTg-AD mice precede inflammation (in preparation)
2. **Caldeira C**, Cunha C, Vaz AR, Falcão AS, Barateiro A, Seixas E, Fernandes A, Brites D. Key aging-associated alterations in primary microglia response to beta-amyloid stimulation. Front Aging Neurosc. (submitted)
3. **Caldeira C**, Oliveira AF, Cunha C, Vaz AR, Falcão AS, Fernandes A, Brites D. Microglia change from a reactive to an age-like phenotype with the time in culture. Front. Cell. Neurosci. 2014; 8:152.

Abstracts in international journals

1. Fernandes A, **Caldeira C**, Falcão AS, Vaz AR, Brites D. (2015) Temporal gene expression profile related to microglia reactivity in 3xTgAD mice. GLIA, 63 Suppl1:E367.
2. **Caldeira C**, Frederico A, Vaz AR, Fernandes A, Brites D. (2013) Microglia reactivity to amyloid-β (Aβ) peptide changes according to experimental ageing. GLIA, 61 Supple1:S49-S216
3. **Caldeira, C.**, Frederico, A., Vaz, A., Fernandes, A., and Brites, D. (2013) Age-related differences in microglia reactivity: relevance to Alzheimer's disease. J. Neurochem.125 (Suppl. 1), 185.

Invited lectures

1. **Caldeira C**, Frederico A, Vaz AR, Fernandes A, Brites D. Age-dependent changes in microglia reactivity to β-amyloid oligomers. Lisbon Area of Neuroscience Meeting. Instituto de Medicina Molecular, Lisbon, Portugal, February 18, 2013.

Communications in scientific meetings

1. **Caldeira C**, Frederico A, Cunha C, Vaz AR, Falcão AS, Barateiro A, Seixas E, Fernandes A, Brites D. Aβ treatment promotes microglia desensitization and induces multiple phenotypes. XIX National Congresso f Biochemistry, Guimarães, Portugal, December 8-10, 2016.

2. **Caldeira C**, Cunha C, Vaz AR, Falcão AS, Fernandes A, Brites D. Altered glial response at early-Alzheimer's disease stage of the 3xTg-AD mice. 2nd Meeting of the PhD Students from the Mind-Brain Doctoral College of the University of Lisbon, Lisbon, Portugal, November 28-29, 2016.
3. **Caldeira C,** Frederico A, Cunha C, Vaz AR, Falcão AS, Barateiro A, Seixas E, Fernandes A, Brites D. Aged microglia show loss of funcion and irresponsiveness to Aβ interaction, while adopting multiple phenotypes. 1st Symposium on Aging Research @CNC, Coimbra Portugal, September 30, 2016.
4. Fernandes A, Monteiro M, **Caldeira C**, Brites D. Neuroblastoma cells expressing amyloid-beta peptide change human microglia microRNA profile and phenotype. 30ª Reunião do Grupo de Estudos do Envelhecimento Cerebral e Demência, Lisbon, Portugal, June 3-4, 2016.
5. Fernandes A, **Caldeira C**, Cunha C, Vaz AR, Brites D. Deregulation of microglia function in the early disease stage of 3xTgAD mice. 29ª Reunião do Grupo de Estudos do Envelhecimento Cerebral e Demência, Aveiro, Portugal, May 29-30, 2015. [Sinapse1(15):233]
6. **Caldeira C**, Vaz AR, Falcão AS, Cunha C, Fernandes A, Brites D. Senescent-related biomarkers are identified in microglia treated with Amyloid-beta peptide. XVIII Congress of the Portuguese Biochemical Society, Hotel Vila Galé, Coimbra, Portugal, December 17-20, 2014.
7. **Caldeira C**, Cunha C, Vaz AR, Falcão AS, Fernandes A, Brites D. Amyloid-beta peptide as an inducer of microglia senescence. 28ª Reunião do Grupo de Estudos do Envelhecimento Cerebral e Demência, Aveiro, Portugal, June 27-28, 2014. [p.4]
8. **Caldeira C**, Vaz AR, Fernandes A, Brites D. Microglia reactivity to Aβ may change with ageing. 27ª Reunião do Grupo de Estudos de Envelhecimento Cerebral e Demência, Coimbra, Portugal, June 28-29, 2013.
9. **Caldeira C**, Frederico, A, Oliveira, AF, Lidónio G, Vaz A, Fernandes A, Brites D. Cell ageing effects on microglia response to Aβ. XIII Reunião da Sociedade Portuguesa de Neurociências (SPN), Luso, Portugal, June 1, 2013. [FIIIB2]
10. **Caldeira C**, Frederico A, Vaz A, Fernandes A, Brites D. Aged microglia are less sensitive to Aβ soluble oligomers. International Society for Neurochemistry (ISN) Satellite Meetings: Understanding Glial Cell Function in the Normal and Injured CNS, Merida, Mexico, April 17-19, 2013.
11. **Caldeira C**, Frederico A, Vaz AR, Fernandes A, Brites D. Age-dependent changes in microglia reactivity to β-amyloid oligomers. Lisbon Area of Neuroscience Meeting. Instituto de Medicina Molecular, Lisbon, Portugal, February 18, 2013.

12. **Caldeira C**, Frederico A, Vaz AR, Fernandes A, Brites D. Aging effects on microglia reactivity to β-amyloid oligomers (AβO). 4th Postgraduate iMed.ULisboa Students Meeting, Faculdade de Farmácia, Universidade de Lisboa, Lisbon, Portugal, December 20, 2012.
13. **Caldeira C**, Frederico A, Vaz AR, Fernandes A, Brites D. Loss of microglia neuroprotective properties with aging: relevance to Alzheirmer's disease. 26ª Reunião do Grupo de Estudos de Envelhecimento Cerebral e Demência, Tomar, Portugal, June 29-30, 2012.

Abstracts in meeting proceedings

1. **Caldeira C**, Cunha C, Vaz AR, Falcão AS, Fernandes A, Brites D. Reduced microglial responsiveness in the early-AD stage of 3xTg-AD mice precede inflammation. 8th iMed.ULisboa Postgraduate Students Meeting & 1st i3DU Meeting, Faculdade de Farmácia, Universidade de Lisboa, Lisbon, Portugal, July 14-15, 2016. [PC28, p.56]
2. **Caldeira C**, Cunha C, Vaz AR, Falcão AS, Fernandes A, Brites D. Microglia polarization subtypes in *in-vivo* and *in-vitro* models of Alzheimer's disease: Association to disease progression and Aβ aggregated species. Encontro Ciência 2016 – Science and Technology in Portugal Meeting, Lisboa Congress Center, Lisbon, Portugal, July 4-6, 2016.
3. **Caldeira C**, Cunha C, Vaz AR, Falcão AS, Fernandes A, Brites D. Suppressed microglial function at early-Ad stage of the triple transgenic mice precede the inflammatory signaling pathways. 1st Meeting of the PhD Students from the Mind-Brain Doctoral College of the University of Lisbon, Lisbon, Portugal, December 2, 2015. [P18, p.44]
4. **Caldeira C**, Cunha C, Falcão AS, Vaz AR, Fernandes A, Brites D. Profile of microglial reactivity in a 3xTg-AD mice model. 7th Postgraduated iMed.ULisboa Students Meeting, Faculdade de Farmácia, Universidade de Lisboa, Lisbon, Portugal, July 15-16, 2015. [PC31, p.54]
5. Monteiro M, **Caldeira C**, Brites D, Fernandes A. Human microglia phenotype and microRNA profile change overtime under amyloid-beta-enriched conditions. 7th Postgraduated iMed.ULisboa Students Meeting, Faculdade de Farmácia, Universidade de Lisboa, Lisbon, Portugal, July 15-16, 2015. [PC35, p.58]
6. **Caldeira C**, Cunha C, Vaz AR, Falcão AS, Fernandes A, Brites D. Microglia senescence by Amyloid-beta peptide. 6th Postgraduated iMed.ULisboa Students Meeting, Faculdade de Farmácia, Universidade de Lisboa, Lisbon, Portugal, July 2, 2014. [PC30, p.49]

7. **Caldeira C**, Vaz AR, Fernandes A, Brites D. Ageing microglia show increased dysfunctionality by amyloid-β peptide when compared to young cultured cells. 5th

Postgraduate iMed.ULisboa Students Meeting, Faculdade de Farmácia, Universidade de Lisboa, Lisbon, Portugal, July 18, 2013. [PC11, p.30]

8. Lidónio G, **Caldeira C**, Vaz AR, Santos M, Moreira R, Falcão AS, Brites D. Exploring a therapeutic approach to prevent Abeta-indiced dysfunctional microglia. 5th Postgraduate iMed.ULisboa Students Meeting, Faculdade de Farmácia, Universidade de Lisboa, Lisbon, Portugal, July 18, 2013. [PC16, p.35]
9. Frederico A, **Caldeira C**, Vaz AR, Fernandes A, Brites D. Age-related differences in microglia reactivity to direct treatment with β-amyloid peptide and to conditioned media from Aβ-treated hippocampal neurons. 8th FENS Forum of Neuroscience, Barcelona, Spain, July 14-18, 2012. [p059.1]
10. **Caldeira C**, Frederico A, Vaz AR, Fernandes A, Brites D. Effects of β-amyloid peptide and conditioned media from Aβ-treated neurons in young vs. aged microglia. 2º Encontro de Biologia Molecular em Saúde, Escola Superior de Saúde Egas Moniz, Monte de Caparica, Portugal, March 9-10, 2012. [p.27]

Abstract

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by a progressive cognitive decline, and accumulation of amyloid-β (Aβ) in senile plaques that are associated with inflammatory molecules released by activated glial cells. Among glial cells, microglia that constitute the intrinsic defense system within the central nervous system, may become activated by Aβ aggregates and develop critical neuroprotective and neurotoxic functions with implications in AD onset and progression. Actually,inflammation has been associated with AD, although nonsteroidal anti-inflammatory drugs have not proven efficacy in halting the progression of the disease, reason why they are indicated as beneficial only in the very early stages of AD. Therefore, features of neuroinflammation and associated microglia phenotypes are still open questions in the understanding of AD pathogenesis and neurodegeneration. Another important issue is the association of AD with ageing and the observation of few microglia and accumulation of dystrophic/desensitized microglia in samples from AD old subjects, strongly suggesting their progressive degeneration and diminished replenishment. Investigation of the multiple activated states of microglia when stressed by Aβ, in particular the increased inflammatory status of microglia with aging, referred as primed reactive, or sensitized cell, or in opposite the proposed state of irresponsive aged microglia, are challenged issues once there are no appropriate procedures to isolate degenerative and senescent microglia for experimentation.

Therefore, the global aim of this thesis was to explore how different microglia phenotypes and ageing may influence Alzheimer's disease (AD) pathogenesis and neuroinflammation, by unraveling their associated neuroprotective and neurotoxic functions in *in vitro* and *in vivo* models. To achieve these objectives, we first (i) developed an experimental model to naturally age primary microglia, which allowed the evaluation of microglia defensive mechanisms (e.g. migration, phagocytosis, autophagy), of a panoply of inflammatory mediators and of senescence-associated markers, in an acutely isolated and activated microglia [2 days *in vitro* (2 DIV)] and an aged cell culture (16 DIV). With the characterization of such differently aged microglia, we (ii) progressed towards the assessment of their responsiveness when treated with 1000 nM of $A\beta_{1-42}$ for 24 h. Finally, in an attempt to translate our *in vitro* research into the triple transgenic AD (3xTg-AD) mice model, we decided (iii) to explore the inflammatory status of the hippocampus and cortex in animals with 3-, 6- and 9 months-old, by assessing microglia phenotypes, as well as the expression of inflammatory cytokines and microRNAs.

We observed in our first study, already published (Caldeira et al Front Cell Neurosci 2014), that (i) in vitro aged microglia switch from a predominant reactive phenotype into cells that although not showing decreased survival, revealed increased dormancy, with morphometric features characteristic of ramified morphology, together with compromised migration, impaired autophagy, reduced phagocytosis, decreased expression of inflamma-miRNAs, and increased presence of senescence-associated markers. In the second study, recently submitted to publication by invitation (Caldeira et al Front Aging Neurosci), using the ageing microglia model and $A\beta_{1-42}$ treatment, we observed that (ii) Aβ treatment caused soma volume increase and process shortening compatible with activated microglia, in both 2 DIV and 16 DIV cells, together with impairment of neuroprotective functions, namely phagocytosis and migration abilities, as well as autophagy, in *in vitro* aged microglia. Interestingly, Aβ led to an increased expression of senescence-like associated markers in 2 DIV microglia, similarly to those of the aged cells. Age-dependent changes included the decrease in the expression of inflammatory mediators and surface receptors, together with the reduction of CD11b+ cells and gain of CD86+ microglia and downregulation of miR-155 and miR-124. Lastly, in our *in vivo* studies at the early-AD stage in the 3xTg-AD at 3 months-old, when Aβ accumulates intraneurally, we observed a downregulation of some activated microglia markers, as well as both typical M1 pro-inflammatory and M2 anti-inflammatory/damage resolution markers. Interestingly, miR-155 revealed to be early upregulated and its increase was sustained at 9 months-old, when extracellular Aβ accumulation is an AD hallmark. At this stage, increase of HMGB1 and decrease of both miR-146a and miR-124 expression is apparent. Curiously, when looking at miR-155 target gene expression we observed new immune-associated molecules that were differently expressed in the 3xTgAD animals by comparison with the wild type mice, both at 3-months and 9-months of age, which will be the subject of study in future works.

We may then conclude that the aged *in vitro* microglia model is very suitable to unravel microglia phenotypic alterations that may explain different cell reactivity in neurodegenerative disorders associated with neuroinflammation and diverse states of disease progression, thus requiring diverse disease-modifying therapies depending on the inflammatory status. We further demonstrate that Aβ induce a heterogeneous population of microglia subtypes instead of only M1 and M2 polarization and that their distribution are age-dependent and influenced by microglia activation state. The increased expression of miR-155 in very early stages of AD in the 3xTg-AD animal model, to be confirmed in AD patients, may additionally reveal as a sensitive biomarker with predictive value if detected in the peripheral blood. The work developed in the present thesis contributed to better define microglial activation phenotypes, in particular

the notion of “good” or “bad” states during AD pathogenesis, while identified new targets to be modulated and assessed as predictive biomarkers, with potential relevance for diagnosis and therapeutic tools for developing innovative medicines.

Keywords**:** Alzheimer’s disease; amyloid-β peptide; neuroinflammation; 3xTg-AD; microglia phenotypes.

Resumo

A Doença de Alzheimer (DA) é considerada como a causa mais comum de demência na população idosa, sendo o envelhecimento apontado como o maior fator de risco associado a esta doença. A DA é uma doença neurodegenerativa que afeta regiões do cérebro relacionadas com a memória e funções cognitivas, levando à incapacidade de estabelecer e manter memórias recentes, a um estado de confusão e à deterioração da linguagem. Esta alteração é progressiva e irreversível tendo como consequência a incapacidade de realizar tarefas diárias, culminando na total dependência de terceiros que se traduz num impacto significativo para os doentes, familiares, cuidadores e sociedade em geral. Atualmente, não existe um tratamento eficaz para curar ou atenuar a progressão da DA.

A DA pode ser classificada em dois tipos com base na idade em que se dá o início da doença: esporádica e familiar. A DA esporádica é a forma mais comum da doença, com um início tardio, afetando normalmente adultos com mais de 65 anos, que podem ou não ter antecedentes familiares desta doença. Até à data, não existem dados que comprovem a existência de hereditariedade na DA esporádica, no entanto o gene *ApoE4* tem sido associado a uma maior probabilidade para algumas pessoas desenvolverem esta forma de doença. A DA familiar é a forma menos comum, onde a doença é transmitida entre gerações e afeta habitualmente indivíduos entre os 40 e os 60 anos de idade.

O cérebro dos doentes de Alzheimer é caracterizado por uma grande atrofia que pode ser causada por alterações neuropatológicas, tais como a acumulação extracelular de placas senis do péptido β-amilóide (Aβ), a formação de tranças neurofibrilares de depósitos da proteína tau hiperfosforilada, a perda de comunicação entre os neurónios (perda de sinapses) e a morte neuronal, que ocorrem em regiões específicas do cérebro. As zonas mais afetadas pela DA são o hipocampo, relacionado com a memória, e os lobos frontal e temporal, relacionados com a linguagem e outras funções cognitivas. De facto, vários estudos na zona do hipocampo e do córtex entorrinal, regiões habitualmente relacionadas com o desenvolvimento precoce da patologia da DA, apontam para que a deposição intraneuronal de Aβ preceda a sua acumulação extracelular, sugerindo ser um acontecimento precoce na patogénese da DA e contribuindo para a progressão desta doença. Outra característica frequentemente observada em cérebros autopsiados de doentes com DA é a neuroinflamação, principalmente em regiões que evidenciam elevados níveis de patologia da doença. No entanto, a neuroinflamação pode estar associada a efeitos benéficos ou prejudiciais ao

nível neuronal. Assim, enquanto uma resposta neuroinflamatória aguda pode ser benéfica para o sistema nervoso central, pois promove a reparação do tecido danificado, uma neuroinflamação continuada em resposta ao estímulo inicial pode originar neurodegeneração e consequentemente disfunção cerebral. A neuroinflamação parece ter um início precoce na DA sendo acompanhada por uma ativação de diversas vias inflamatórias, habitualmente associadas à ativação das células gliais. De facto, a gliose é uma característica neuropatológica frequentemente observada em cérebros de doentes com DA, em que a microglia responde através da alteração do seu fenótipo, produzindo diversos mediadores pro-inflamatórios, e até anti-inflamatórios, criando um ciclo de auto-perpetuação do quadro neuroinflamatório que contribui para o cenário de inflamação crónica observado na DA. Esta ativação continuada da microglia, associada a uma disfunção associada ao envelhecimento e ao estímulo tóxico, contribui para o início e progressão da DA. Modificações dos fenótipos microgliais, ocorrendo de forma diferente na população de células de um determinado local, ou mesmo de forma diversa entre as várias regiões do encéfalo, podem estar na origem do fracasso do tratamento com medicamentos anti-inflamatórios não esteróides como prevenção da DA. De facto sabe-se hoje que apenas são eficazes na prevenção da progressão da doença quando iniciadas, ou em curso, na fase pré-clínica da mesma e que até poderão ser prejudiciais nas fases mais avançadas, onde até a inflamação tem vindo a ser referida como já não tendo lugar.

Esta tese teve como objetivos: (i) desenvolver um modelo experimental *in vitro* de células de microglia capaz de reproduzir uma célula ativada (mimetizando a existência de inflamação) e uma célula envelhecida em cultura (mais senescente) que permitisse caracterizar o seu diferente comportamento funcional e traçar a sua assinatura de reatividade (**Capítulo 2**); (ii) identificar a resposta dos fenótipos ativados e mais senescentes da microglia a uma concentração não tóxica de Aβ e estabelecer os seus perfis de resposta ao estímulo (**Capítulo 3**); e (iii) definir o contexto inflamatório e as subpopulações de microglia na região cortical e hipocampal do cérebro de ratinhos do modelo animal triplo transgénico para a DA, o 3xTg-DA, em 3 períodos bem definidos (3-, 6- e 9 meses de idade) e correspondentes a diversas fases de progressão da doença (**Capítulo 4**).

Este estudo iniciou-se, então, com o desenvolvimento e estabelecimento dos modelos que melhor definissem a microglia ativada (decorrente do processo de isolamento) e a envelhecida em cultura, manifestando marcadores associados a senescência celular. Para tal, as células foram isoladas a partir de cérebros de ratinhos CD1 recém-nascidos, tendo sido mantidas até 2 dias *in vitro* (DIV) (“ativadas”) ou até 16 DIV em cultura (“envelhecidas”), tendo sido analisadas, quer relativamente à existência

de marcadores inflamatórios, quer quanto às suas propriedades funcionais. As células com 2 DIV, tal como esperado, apresentaram uma morfologia mais amebóide que se alterou para uma morfologia mais ramificada, havendo também diminuição da expressão de marcadores característicos de fenótipo mais reativo, com o tempo em cultura. Além disso, observámos ocorrer perda de funcionalidade microglial com diminuição da capacidade de migração, autofagia e fagocitose por parte destas células envelhecidas em cultura. É interessante assinalar que dos 2 DIV para os 16 DIV se verificou haver ativação da metaloproteinase (*matrix metalloproteinase*, MMP)-2 que se acompanhou de uma descida da MMP9, juntamente com uma redução da libertação de glutamato, da ativação do fator nuclear kappa-B, e da expressão dos recetores *Toll-like* (TLR)-2 e TLR4. Curiosamente, verificou-se ainda uma redução na expressão dos *microRNA* (miR)-124 e miR-155, acompanhada por um aumento de marcadores associados à senescência, como a de beta-galactosidade associada à senescência e de expressão do miR-146a. Estes resultados indicam que as células microgliais jovens (2 DIV) têm uma resposta mais reativa, enquanto que com o aumento da idade *in vitro* as células microgliais (16 DIV) tendem a perder a sua capacidade de resposta e ficar mais senescentes.

O estabelecimento deste modelo de microglia ativada e envelhecida, permitiu-nos, então, avaliar a sua resposta diferencial ao estímulo de concentrações não tóxicas do péptido $A\beta_{1-42}$. Para isso, células microgliais com 2 e 16 DIV foram expostas durante 24 horas a 50 nM e 1000 nM do péptido $A\beta_{1-42}$, após o que se avaliou a capacidade de resposta ao estímulo. Verificámos novamente que algumas das funções neuroprotetoras desempenhadas pela microglia, como a fagocitose, migração e autofagia, se encontravam comprometidas com o envelhecimento *in vitro*. Por outro lado, observámos que a exposição das células ao Aβ desregulou a capacidade fagocítica da microglia de 2 DIV, ao mesmo tempo que estimulou a autofagia e contribuiu para o aumento dos marcadores senescentes. Contudo, foi evidente a resposta da microglia de 2 DIV ao Aβ em termos do aumento da expressão de mediadores inflamatórios, tais como citocinas pró-inflamatórias (TNF-α, IL-1β e IL-6), proteínas relacionadas com o inflamasoma (HMGB1, IL-18 e NLRP3) e recetores reguladores (TLR2, TLR4 e CX3CR1). Esta reatividade foi contudo significativamente diminuída com o tempo em cultura, ocorrendo redução da expressão de miRs relacionados com a inflamação (miR-155 e miR-124) e marcadores de microglia reativa (células $CD11b^{+}$). Curiosamente, observámos que o tratamento com Aβ foi capaz de induzir a polarização das células tanto para o fenótipo M1 (iNOS e MHC class II), como para o fenótipo M2 (Arginase 1 e TGF-β) nas células de 2 DIV e 16 DIV. Os resultados mostram, assim, que a exposição ao Aβ leva a uma heterogeneidade de fenótipos microgliais, incluindo o

típico M1/pró-inflamatório, o M2/anti-inflamatório/resolução do dano e o mais distrófico/senescente associado à perda da capacidade de resposta, com uma distribuição que se verificou depender do estado de ativação da célula aquando do estímulo.

Por fim, para melhor perceber os fenótipos microgliais no contexto do parênquima cerebral em estádios diferentes da doença, em particular nos mais precoces e nas regiões do hipocampo e do córtex fomos avaliar, em ratinhos 3xTg-DA, os marcadores inflamatórios aos 3 meses de idade, onde se verifica a oligomerização do Aβ intraneuronal, aos 6 meses onde se inicia a acumulação de placas no exterior da célula e aos 9 meses, correspondentes a uma fase mais avançada da doença com evidente acumulação de Aβ extracelular. Curiosamente, aos 3 meses observámos uma subexpressão de alguns marcadores de ativação da microglia (TNF-α, IL-1β, IL-6, IL-18 e HMGB1), tanto do tipo M1 pró-inflamatórios (MHC class II e CEBP-α), como de M2 anti-inflamatórios e/ou relacionados com a resolução do dano (SOCS1, TGF-β e Arginase 1), sugerindo que haja uma tentativa de prevenir o dano e a expressão de proteínas mal-formadas. Aos 6 meses verificámos não haver alterações assinaláveis, uma vez que corresponde ao período onde se verifica uma diminuição da acumulação do Aβ intraneuronal sem grande acumulação extracelular, pelo que decidimos não incluir os resultados obtidos na presente tese. No entanto, ao avaliar os resultados obtidos aos 9 meses, verificámos um aumento da expressão de alguns marcadores inflamatórios (TNF-α, IL-1β e HMGB1), e típicos de um fenótipo M1 (iNOS) e M2 (SOCS1 e Arginase 1). Verificámos ainda uma elevada expressão do miR-155 logo na fase inicial da doença (3 meses), o qual se manteve elevado aos 9 meses, indicando a sua potencialidade como biomarcador preditivo da DA. Por outro lado, os miR-146a e miR-124, embora elevados aos 3 meses, estavam diminuídos aos 9 meses, que poderá favorecer o ambiente inflamatório nesta etapa. Curiosamente, a análise de alvos do miR-155 identificou a expressão distinta de moléculas relacionadas com a resposta do sistema imunitário, encontrando-se estas menos expressas no modelo 3xTg-DA tanto aos 3 meses, como ainda de forma mais marcada aos 9 meses. Estes resultados demonstram uma resposta imune disfuncional no modelo 3xTg-DA quando comparado com animais controlo da mesma idade.

Concluindo, conseguimos desenvolver e estabelecer um modelo celular de dois fenótipos microgliais, com comportamento que mimetiza a microglia mais ativada e mais envelhecida. O modelo foi útil para o estudo do efeito do Aβ em ambas as situações e poderá ajudar a perceber a disfuncionalidade da microglia pelo envelhecimento, bem como testar e identificar moléculas capazes de recuperar a funcionalidade

neuroprotetora da microglia. Os nossos resultados mostraram que o péptido Aβ poderá contribuir para a perca da funcionalidade da microglia quando num ambiente inflamatório, envelhecendo-a de forma prematura e indicando que a imunossupressão pode estar relacionada com o surgimento da DA associada à idade, e que a presença de Aβ poderá contribuir para a perca da funcionalidade da microglia e favorecer a progressão da doença. Os estudos realizados permitiram evidenciar igualmente, que a microglia assume diversos fenótipos quando estimulada pelo Aβ e que a microglia mais envelhecida se torna incapaz de combater o dano. Isto pode, em parte, explicar o insucesso das terapias anti-inflamatórias utilizadas para combater a DA. No modelo *in vivo*, o miR-155 revelou ser um potencial biomarcador precoce da inflamação na DA. Por outro lado, a resposta atenuada da microglia na fase inicial da doença, pode estar associada à contenção do dano causado pelo Aβ intraneuronal. Com a acumulação do Aβ a nível extracelular, e apesar da perca das funcionalidades neuroprotetoras associadas à migração e fagocitose, a microglia torna-se mais reativa. Posteriormente, parece entrar numa fase de maior latência o que se poderá traduzir num ambiente mais favorável à progressão da doença e exigir terapêuticas de "rejuvenescimento" da célula. Estes dados reforçam a existência de diversas fases de progressão patológica da DA, bem como de distintos subtipos de fenótipos constituindo uma população microglial heterogénea e influenciável pelo ambiente patológico que a envolve, o que nos remete para a necessidade de utilização de diferentes estratégias terapêuticas consoante o estadio e as alterações de natureza inflamatória que lhe estejam associadas.

Palavras-chave: Doença de alzheimer; péptido β-amilóide; neuroinflamação; 3xTg-DA; fenótipos da microglia.

Chapter 1

GENERAL INTRODUCTION

1. Alzheimer's Disease

1.1. Historical perspective

Alzheimer's disease (AD) was discovered in 1906 by Alois Alzheimer, a German psychiatrist and neuropathologist that characterized and described the case of Auguste Deter, a 51-year-old woman who had died of an unusual mental illness (Balducci and Forloni, 2011). Her symptoms included memory loss, confusion, hallucinations, disorientation, language problems, and unpredictable behaviour, which are several of the fundamental features observed in most patients nowadays (Balducci and Forloni, 2011). Autopsy of Auguste brain revealed changes in the brain tissue like a highly shrunken brain, dying cells and the presence of two kind of aggregates, plaques and tangles, that have become hallmarks of the disease (Kidd, 2008).

About one hundred years after this discovery, AD became one of the most common neurodegenerative diseases and the most prevalent cause of dementia among old people (Khairallah and Kassem, 2011). Estimates vary, but is suggested that more than 5 million people in the Unites States (Huynh et al., 2017), and more than 35 million worldwide (Isik, 2010), may be affected by AD. Meanwhile, due to the increase in population life expectancy this number is expected to triple by 2050 (Bettens et al., 2013; Hebert et al., 2003). However, a recent report where dementia prevalence among 65 years or older were compared between 2000 and 2012 showed a reduction from 11.6% to 8.8%, and associated to increased educational attainment, although there is still the need to evaluate other social, behavioural, and medical factors (Langa et al., 2016).

AD is a neurodegenerative disorder characterized by subacute chronic inflammation, neurotoxicity, oxidative stress, and reactive gliosis (Lopategui Cabezas et al., 2012), that affect regions of the brain related with memory and cognitive functions, leading to the inability to establish and maintain recent memories, confusion and language deterioration (Rubio-Perez and Morillas-Ruiz, 2012). Symptoms of AD occur for several years to a decade, being mortality often related with resulting secondary issues, such as opportunistic infections or multi-organ failure (Siciliano et al., 2011). The lengthy and progressive cognitive decline seriously interferes with daily life (Khairallah and Kassem, 2011), having a significant impact in patients and their families, caregivers, and society in general (Kidd, 2008). The tremendous need of care required by Alzheimer's patients, in association with the lack of an effective treatment for this disease, makes AD a high financial burden to both families and health systems (Salawu et al., 2011).

Advanced age is the most important risk factor associated with AD, with the incidence of the disease doubling every five years after 65 years of age (Salawu et al.,

2011). AD can be classified either as late-onset AD (LOAD or sporadic) or early-onset AD (FAD or familial), based on the age of first signs of disease (Kidd, 2008). The late-onset AD is the most common form of the disease, comprising about 90-95% of cases, and usually occurs 65 years of age. In contrast, early-onset AD is relatively rare, arising in people aged 40-60, and represents the other 5-10% of AD cases (Harman, 2006). Some FAD cases are caused by inherited change in one of three genes: the *amyloid precursor protein* (*APP*) gene on chromosome 21, the *presenilin 1* (*PS1*) gene on chromosome 14, and the *presenilin 2* (*PS2*) gene on chromosome 1 (Salawu et al., 2011). LOAD develops without any specific and known cause, showing no significant genetic link, with the exception of *apolipoprotein E* (*ApoE*) gene carrying the ε4 allele (*ApoE4*), which appears to be a major genetic susceptibility AD risk factor (Kidd, 2008).

Although AD has been traditionally associated with ageing, recent approaches refer an early origin. Indeed, abnormally phosphorylated tubulin-associated unit (tau) protein was recently identified in brains of people aged between 4 and 29 years (Braak and Del Tredici, 2011), suggesting that AD-related pathological process may begin before puberty or in early young adulthood.

1.2. Pathophysiology and histopathological findings

A wide atrophy of the brain is observed in AD patients due to a high number of neuropathological modifications, like abnormal deposition of amyloid plaques, formation of neurofibrillary tangles, loss of connections between neurons, and neuronal death (**Figure 1.1**). Nevertheless, these modifications in the brain are not evenly spread throughout the brain (Wenk, 2006). Indeed, preferentially affect specific brain areas which are essentially consistent from patient to patient (Braak and Braak, 1991). Although the size, shape, and distribution pattern of amyloid aggregates vary from individual to individual, its onset usually begins in the basal portions of the isocortex, spreading then to the areas in association with isocortex, and finally reaching the hippocampal region. In contrast, neurofibrillary tangles occur according to a set pattern, first affecting the entorhinal cortex, following the hippocampal neurons and, finally, extending to surrounding neurons in extra hippocampal regions, like isocortical areas (Braak and Braak, 1991).

The neuronal damage appears to be caused by changes that occurs both inside and outside the neuron, and is characterized by the presence of intracellular both amyloid-β (Aβ) peptide and filamentous deposits of tau protein, named neurofibrillary tangles (NFTs), as well as by extracellular aggregates of Aβ, named amyloid plaques (Bayer and Wirths, 2010; Sheng et al., 2012) (**Figure 1.1C**). Emerging evidences link the intraneuronal accumulation of Aβ to synaptic dysfunction, cognitive dysfunction and

formation of plaques in AD (Gouras et al., 2005; Gouras et al., 2010; Wilson et al., 2016). The accumulation of all these pathological materials in the brain is considered the main histopathological hallmarks of AD. In fact, although healthy brains also display amyloid and tangles, mainly with aging and the presence of the *ApoE4* gene, the AD brains effectively have a higher number of plaques and tangles (Kidd, 2008). Furthermore, the increased number of reactive microglia and astrocytes close to site of injury, called gliosis, is also observed in AD brain (Rubio-Perez and Morillas-Ruiz, 2012) (**Figure 1.1C**). Another feature of the disease is the malfunctioning of once-healthy neurons, selectively in the limbic system and neocortex, losing connections with other neurons, and dying. This synaptic loss and selective neuronal death is currently the one that best correlate with cognitive deficits observed in AD (Bi, 2010; Isik, 2010).

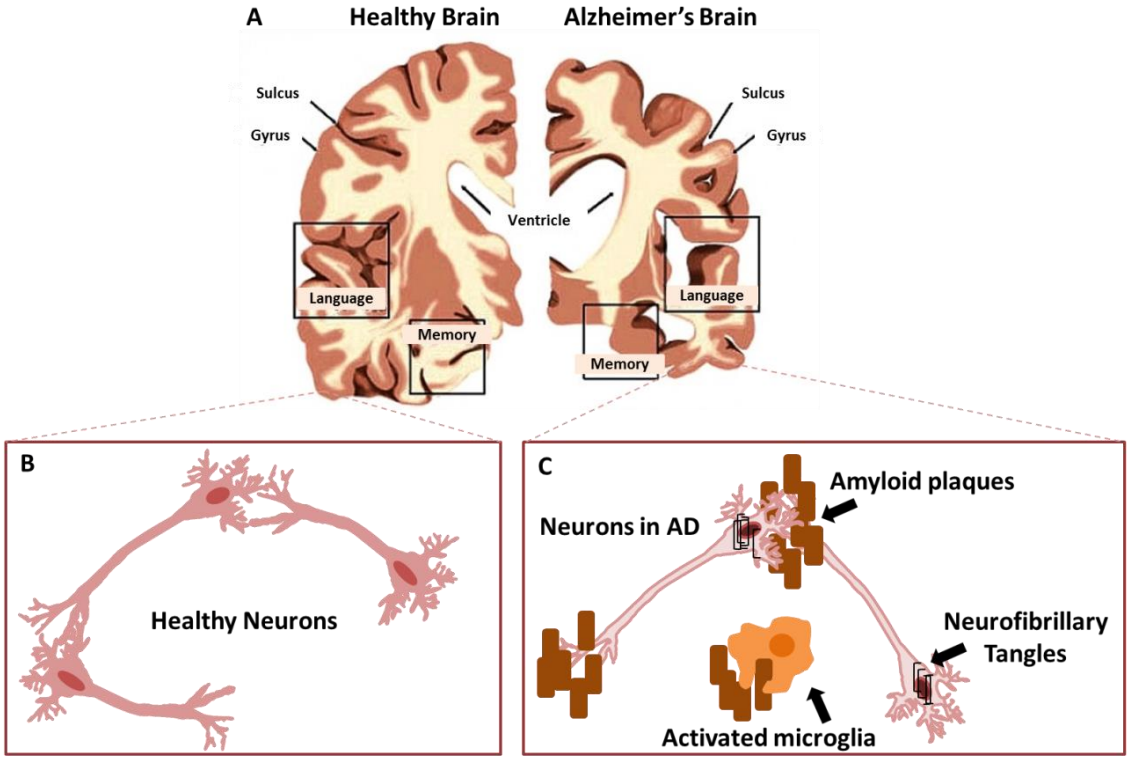

Figure 1. 1 – Alzheimer's disease neuropathology. (A) An overall shrinkage of brain tissue is observed in later stages of Alzheimer's disease (AD), accompanied by extended sulci, narrowed gyri and enlarged ventricles. Temporal lobes are the most affected. Short-term memory begins to fail when hippocampal neurons, forming part of the limbic system, degenerate. Due to the spread of the disease through the cerebral cortex, the ability to perform daily tasks declines and language becomes impaired. **(B)** In healthy brain, features of the disease are generally absent, although they can be observed as a result of ageing. **(C)** Neurofibrillary tangles are found within a large number of degenerated neurons, as well as extracellular amyloid plaques which are often surrounded by activated microglial cells. Adapted from picture at BrightFocus Foundation website.

Actually,, there are not a clear understanding of the complex brain changes involved in the onset and progression of AD. In this context, research has focused on

the various mechanisms that are associated with this disease, such as Aβ aggregation and neurotoxicity, tangle formation by hyperphosphorylated tau protein, neuroinflammation, oxidative stress and mitochondrial dysfunction.

1.2.1. Amyloid-β peptide

According to the amyloid hypothesis, deposition of Aβ peptide in the brain is the triggering of AD pathogenesis due its neurotoxicity (Cavallucci et al., 2012; Puzzo and Arancio, 2013). Aβ is a hydrophobic protein derived from proteolytic cleavage of APP, which is a transmembrane protein located on the plasmatic membrane, or in intracellular compartments, of neurons and glial cells that exist in high quantity in the brain (O'Brien and Wong, 2011; Puzzo and Arancio, 2013). It is known that APP is expressed by neurons in response to cell damage (Rubio-Perez and Morillas-Ruiz, 2012).

The proteolytic cleavage of APP occurs in a series of sequential steps beginning with the cleavage by α- or β-secretase, followed in both cases by cleavage by γ-secretase, originating, or not, various fragments of Aβ peptide with several lengths. APP can be processed by the non-amyloidogenic pathway in which α-secretase, an enzyme from the disintegrin and zinc metalloproteinase (ADAM) family (Allinson et al., 2003), cleaves APP within the Aβ domain, originating a membrane-bound C-terminal fragment of 83 amino acids (C83) and an N-terminal soluble ectodomain fragment (sAPPα), which is released extracellularly. The C83 fragment is further cleaved by γ-secretase giving rise to a short fragment dominated P3. Thus, the Aβ peptide formation is prevented in the non-amyloidogenic pathway of APP processing, since the α-secretase cleavage takes place in Aβ sequence **(Figure 1.2)** (Cavallucci et al., 2012). Alternatively, APP can be processed by amyloidogenic pathway, being first cleaved by β-secretase, which acts in the β-site APP-cleaving enzyme 1 (BACE1) (Vassar et al., 1999), producing a membrane-bound C-terminal fragment of 99 amino acids (C99) with a N-terminal soluble ectodomain fragment (sAPPβ) released into the extracellular medium. The C99 fragment starts at residue 1 of Aβ region and the subsequent cleavage by γ-secretase results in the Aβ peptide formation **(Figure 1.2)** (Cavallucci et al., 2012). While α-secretase and β-secretase cleave APP at single sites, γ-secretase, a complex of proteins which uses PS1 and PS2 as catalytic subunits, performs a set of intramembranous cuts, giving rise to products of various lengths (Sheng et al., 2012). Aβ peptide length can vary between 39 and 43 amino acids (Chromy et al., 2003), but most of the Aβ produced is formed by 40 residues ($Aβ_{1-40}$), although a slightly larger variant of 42 residues ($Aβ_{1-42}$) can also be formed (Lewczuk et al., 2004; Wesson et al., 2011). These Aβ peptides can spontaneously self-associate into larger structures, ranging from low molecular oligomers (Aβo) to insoluble aggregates of fibrils (Aβf), until be deposited as senile

plaques **(Figure 1.2)** (Maezawa et al., 2011; Rubio-Perez and Morillas-Ruiz, 2012). However, as $A\beta_{1-42}$ variant is more hydrophobic, it is more conducive to form aggregates (Cavallucci et al., 2012). Although amyloid plaques are formed from several different peptides and proteins (Allsop, 2000), $A\beta_{1-42}$ is the prevalent form in these amyloid deposits and believed to be more neurotoxic than $A\beta_{1-40}$ and other Aβ species (Cavallucci et al., 2012; Maezawa et al., 2011).

The neurodegenerative process of AD is initially characterized by synaptic injury, which is highly correlated to the cognitive impairment of the individuals. Furthermore, several studies support the idea that AD pathology is related to progressive accumulation of Aβ peptide, due to a discrepancy among the levels of Aβ production, aggregation and clearance. While, in all familial forms of AD are observed mutations in *APP* or *PS* genes that result in an increased Aβ production or aggregation, the Aβ accumulation in the sporadic form is considered to be more related with a failure in the clearance mechanisms (Crews and Masliah, 2010). Indeed, Down's syndrome patients who possess an extra copy of chromosome 21, on which the *APP* gene resides, also develop an AD-like pathology corroborating the idea that overexpression of APP causes familial AD (Huang and Mucke, 2012). In turn, an association between Aβ clearance and clearance molecules, such as ApoE, show *ApoE4* as the less efficient in clearing Aβ, reason why is considered as the top sporadic AD gene (Huang and Mucke, 2012; Kim et al., 2009; Liu et al., 2013).

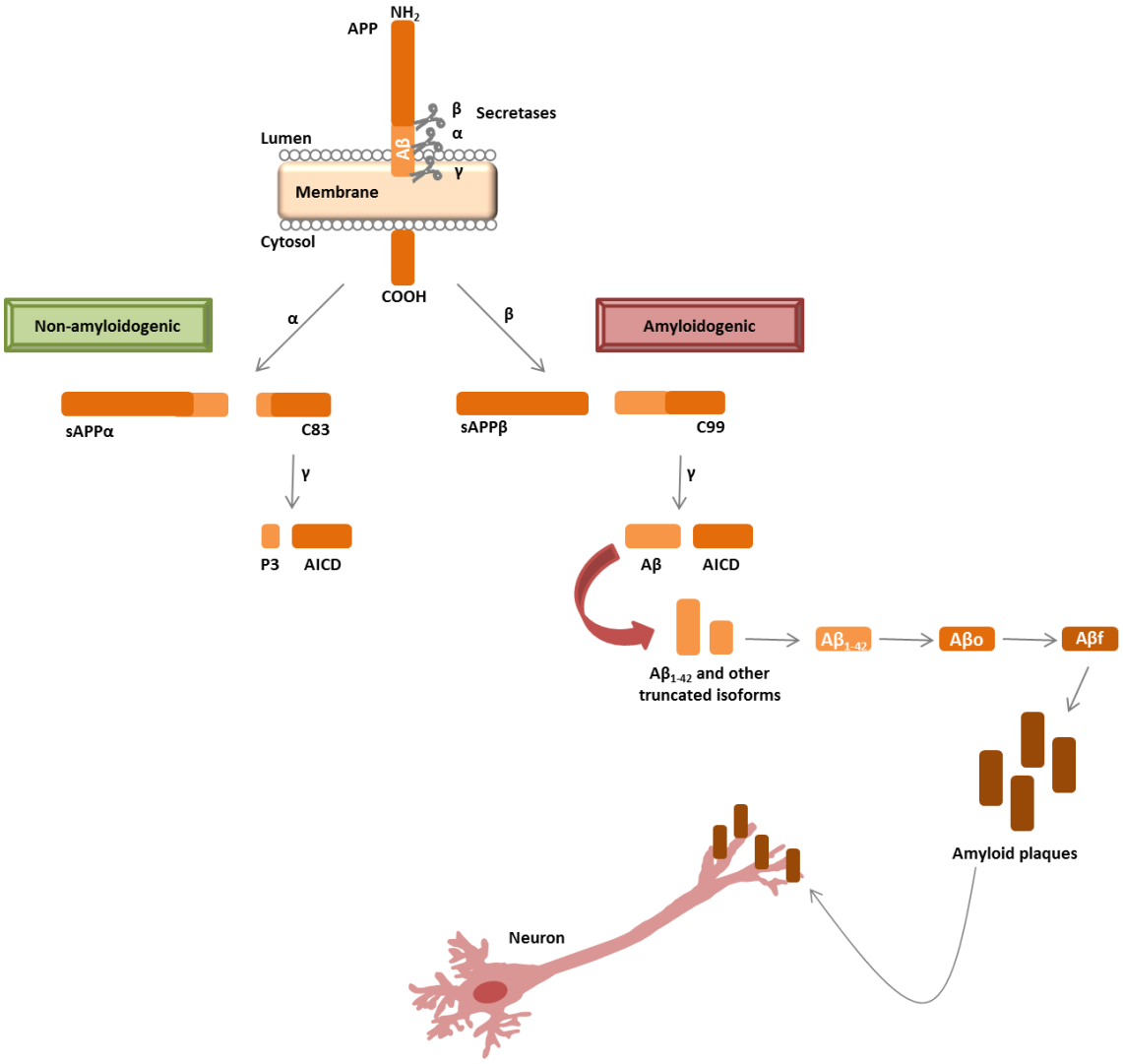

Figure 1. 2 – Proteolytic cleavage of amyloid precursor protein (APP). APP can be processed by two different pathways: the non-amyloidogenic pathway and the amyloidogenic pathway. In the non-amyloidogenic pathway, APP is first cleaved by α-secretase generating a secreted fragment of APP (sAPPα) and a membrane-bound carboxy-terminal fragment of 83 amino acids (C83). Thereafter, C83 is cleaved by γ-secretase, giving rise to P3, avoiding amyloid-β (Aβ) generation. In turn, amyloidogenic pathway is started by β-secretase producing a sAPPβ fragment and a membrane-bound fragment of 99 amino acids (C99), which is cleaved by γ-secretase originating fragments of Aβ peptide with several lengths, which self-aggregates into Aβ oligomers (Aβo), than into amyloid fibrils (Aβf), and finally are deposited as amyloid plaques. These amyloid plaques promote synaptic impairment culminating in neuronal loss. APP intracellular domain (AICD) is an end-product in both pathways, which is translocated into the nucleus to regulate gene transcription.

The role of Aβ in AD pathogenesis is not completely understood, but it is for long assumed that Aβ accumulation is essential for its neurotoxic effects (Walsh and Selkoe, 2007). As one of the hallmarks of AD is the accumulation of extracellular Aβ, the majority of the works have focused on the study of insoluble Aβf, which are the main aggregated

constituent of amyloid plaques (Cavallucci et al., 2012). In addition, as insoluble Aβf aggregates are neurotoxic both *in vitro* and *in vivo* (Walsh et al., 1999), it has long been hypothesized that fibrils represent a primary cause of AD neurodegeneration. Nonetheless, the number of plaques and the levels of insoluble Aβf present in AD brains have poor correlation with local amplitude of the synaptic loss and neuronal death, as well as with disease severity (Bi, 2010; Cavallucci et al., 2012). Furthermore, it was reported the presence of a significant amyloid burden in individuals who do not demonstrate cognitive impairment (Aizenstein et al., 2008; Pike et al., 2007). On the other hand, some studies suggest that the levels of Aβo are really correlated with synaptic dysfunction and cognitive impairment (Walsh and Selkoe, 2007). Aβo isolated from the brains of patients with AD show a wide range of molecular weight, from less than 10 kDa to more than 100 kDa (Bi, 2010). In fact, a high number of different structures of Aβo have been reported, being classified as Aβ dimers and trimers, Aβ-derived diffusible ligands, globulomers, Aβ*56 (56 kDa) and others (Fandrich et al., 2011; Lesne et al., 2006). These diverse soluble oligomeric forms of Aβ were shown to be more toxic than the fibrillar species (Tomic et al., 2009), being considered the most synapto-toxic ones (Crews and Masliah, 2010). Thus, Aβo are pointed out as the principal players in AD onset and neurodegeneration (Cavallucci et al., 2012; Shankar et al., 2008).

Although the classical view is that Aβ is accumulated extracellularly, several studies from transgenic mice and human patients points that this peptide can also deposit intraneuronally, which may be an early event in the pathogenesis of AD and contributory to disease progression (LaFerla et al., 2007). Moreover, intraneuronal Aβ immunoreactivity has been observed in the hippocampus and in the entorhinal cortex, which are brain regions more connected to the development of early AD pathology, preceding Aβ extracellular deposition (Gouras et al., 2000). In addition, it was reported that Aβ oligomerization begins intraneuronally (Walsh et al., 2000). Furthermore, studies in primary cultures of neurons that overexpressed APP corroborate the production of intraneuronal Aβ which induces neuronal apoptosis, and this neurotoxicity seems to be related to the formation of intraneuronal aggregates of $A\beta_{1-42}$ (Nathalie and Jean-Noel, 2008; Octave, 2005). Besides the release of intracellular Aβ as a result of APP cleavage, that can occur in several cellular compartments and be confined to the cell, it can also result from the uptake of extracellular Aβ through receptors or transporters, like the receptor for advanced glycation end products (RAGE) and the α7 nicotinic acetylcholine receptor (α7nAchR) (LaFerla et al., 2007).

Interestingly, the formation of Aβo have been considered to be related to early and central events in the pathogenesis of AD (Shankar et al., 2008), suggesting that their direct effects on neuronal integrity, myelination and synaptic plasticity may be associated to memory problems and cognitive impairment (Hardy and Selkoe, 2002). Moreover, white matter damage and myelin abnormalities in the brain of AD individuals, supported by cytopathological studies (Braak et al., 2000; Kobayashi et al., 2002), suggest the participation of oligodendrocytes in the pathogenesis and progression of AD (Roth et al., 2005). Furthermore, the high levels of Aβ in the white matter of AD patients can damage cholesterol rich membranes, such as those present in oligodendrocytes and myelin (Roher et al., 2002). Also, it was observed that neurons that are myelinated at the latest periods of life are the first to be affected in AD (Braak and Braak, 1996), suggesting that the death of myelinating oligodendrocytes represents an important step in the onset of disease (Bartzokis et al., 2004).

Nevertheless, one must not forget that the cleavage of APP, and subsequent production of Aβ, is a physiological process. This idea is supported by the observation that the Aβ peptide is produced in its soluble form under normal conditions during cellular metabolism (Cavallucci et al., 2012). In addition, several studies suggest physiological properties of Aβ, including neurotrophic and neuroprotective effects, as well as stimulation of neural-progenitor proliferation and modulation of synaptic function, mainly at low concentrations, which although not allowing the formation of oligomers (Chasseigneaux and Allinquant, 2012), may be considered later on a pathological condition when the levels of Aβ increase (Cavallucci et al., 2012).

1.2.2. Tau protein

The presence of intracellular NFTs, constituted mainly by hyperphosphorylated tau protein, is another distinct hallmark in AD histopathology (Isik, 2010; Rubio-Perez and Morillas-Ruiz, 2012). Tau (τ) is a highly soluble protein, member of the family of microtubule-associated proteins (MAPs), synthesized within the neuron, and linearly organized in the axon promoting stability and assembly of microtubules. The binding of tau to microtubules is controlled by its phosphorylation level. Tau protein has the ability to become phosphorylated at multiple sites by several protein kinases (Cdk5, GSk3β, MARK and ERK2), which cause changes in its shape and regulates its biological activity, modulating the capacity to bind to microtubules and causing microtubule depolymerisation (Duan et al., 2012; Simic et al., 2016). In opposite, phosphatases (PP1, PP2, PP2B and PP2C) can reduce the phosphorylation of tau protein and restore its capacity to bind to microtubules. This balance between kinases and phosphatases

activity is interrupted under pathological conditions, on which the increment in the kinase activity and the reduction in the phosphatase activity trigger hyperphosphorylation of tau protein (Duan et al., 2012). In fact, during AD progression the toxicity of tau, Aβo and Aβf could lead to tau-hyperphosphorylation cascade leading to subsequent dissociation from microtubules, with a loss of function, and formation of abnormally twisted filaments that aggregate into NFTs (Anoop et al., 2010; Kidd, 2008) **(Figure 1.3)**. Commonly, NFTs first appear in the entorhinal cortex, spreading to the hippocampus, and then to the neocortex (Kidd, 2008). Also excitotoxicity, oxidative stress and inflammation have been associated, independently or in combination, to protein misfolding of tau, which contribute to an abnormal detachment of tau from microtubules (Duan et al., 2012; Kruger and Mandelkow, 2015). Thus, NFTs are thought to be associated with a collapse of the microtubule network, disturbances of axoplasmic transports, synapse loss, neuritic atrophy, and neuronal death (Octave, 2005). The neuronal death leads to the release of intraneuronal tau, in monomeric or aggregated form, into the extraneuronal space, which can be neurotoxic and trigger the spreading of tau pathology found in the AD brain (Gomez-Ramos et al., 2006). In addition, some studies relate soluble oligomeric species of tau protein as being more damaging to proper neuronal function than insoluble aggregates (Kruger and Mandelkow, 2015), similarly to what occurs with Aβ.

According to the amyloid cascade hypothesis, excessive production of Aβ is the trigger of all other pathological changes, such as gliosis, neuronal death, synaptic dysfunction, as well as development of NFTs **(Figure 1.3)** and dementia in all AD cases (Simic et al., 2016). However, neurofibrillary degeneration of abnormally hyperphosphorylated tau appears to be required for the clinical expression of the disease (dementia). Moreover, while approximately 30% of normal aged individuals have a charge of amyloid plaques in the neocortex similar to that found in typical cases of AD, in several tauopathies neurofibrillary degeneration of abnormally hyperphosphorylated tau without plaques presence is related with dementia. Thus, the tau role in neurodegenerative process is also supported by the correlation of NFTs and the AD onset and progression (Gong et al., 2010). Several studies suggest that Aβ may drive tau pathology, while tau can mediate Aβ neurotoxicity, pointing out a partnership between Aβ and tau in AD pathology (Duan et al., 2012). However, it has not yet been fully elucidated how these two abnormal protein aggregates (Aβ and NFTs) are related in AD pathogenesis (Nicholson and Ferreira, 2009).

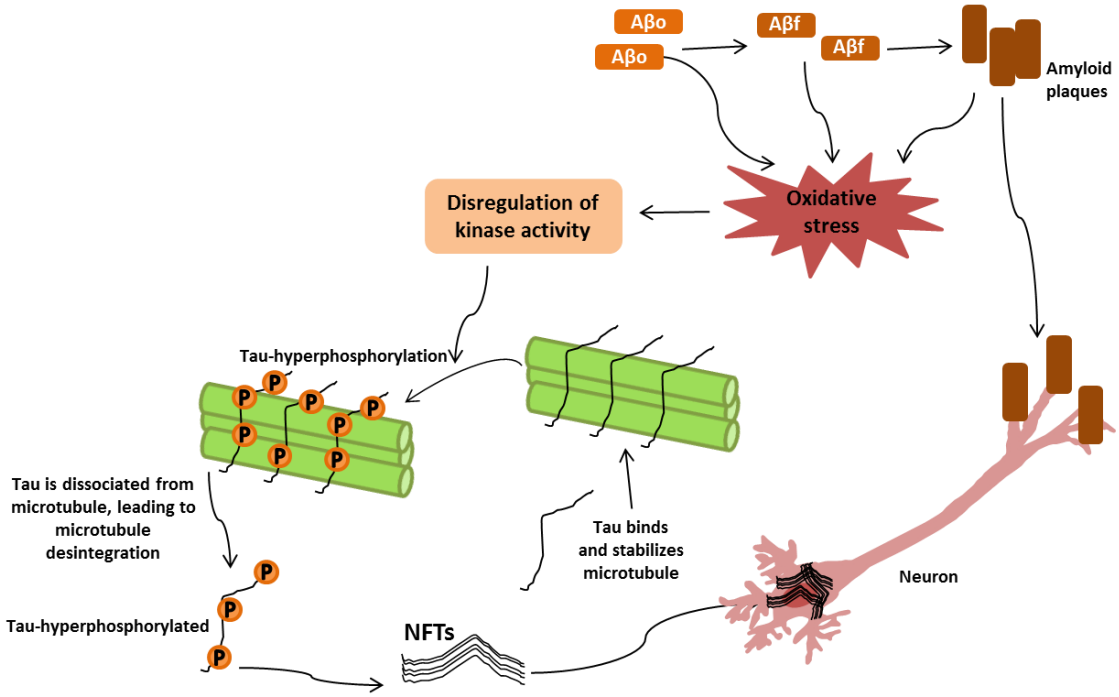

Figure 1. 3 – Tau-mediated neurodegeneration. Tau protein binds to microtubules providing microtubule stability. In pathological conditions, toxic Aβ oligomers (Aβo) and amyloid fibrils (Aβf) may deposit as amyloid plaques triggering the cascade of tau-hyperphosphorylation due to the increase in kinase activity. Upon hyperphosphorylation tau dissociates from microtubules, causing microtubule disintegration, and aggregates into neurofibrillary tangles (NFTs) within neurons. This can promote cytoskeleton destabilization, impaired normal axonal transport, synaptic dysfunction and neuronal death.

1.2.3. Neuronal cell death

It is well known that AD develops after a progressive death of many neuronal cells (neurodegeneration) across large areas of the brain. Over time, as more and more neurons die, both signs and symptoms related with disease become worse (Johnstone et al., 2016). In fact, a massive loss of hippocampal neurons is suggested as a pathological basis for irreversible cognitive commitment in AD patients (Xu et al., 2016). However, the underlying causes and mechanisms of neuronal cell death are not fully understood.

Several different hypotheses have been advocated as a cause of neurodegeneration. One of them, previously mentioned in section **1.2.2.**, is the amyloid cascade hypothesis, which proposes the accumulation of Aβ in the brain as the primary driver to NFTs formation and subsequent neuronal death (Hardy and Selkoe, 2002). An alternative hypothesis suggests that the breakdown of cerebral capillaries as a consequence of ageing causes microvascular hemorrhages, which in turn lead to plaques deposition, NFTs formation, and subsequent neuronal death (Stone, 2008). Another hypothesis is that mitochondrial dysfunction is a major contributor to neuronal death. Mitochondria is considered to have a critical role in brain metabolism, the third

most energy-expensive organ in the human body, controlling cell life and death not only as the energy-generating organelles, but also producing potentially toxic reactive oxygen species (ROS) and replenishing proteins that regulate the intrinsic apoptotic pathway (Onyango and Khan, 2006). In fact, 95% of the cell energy supply is produced in mitochondria (Bonda et al., 2010), being, however, also the major source of ROS generation in the generality of cells (Kim et al., 2015). Indeed, mitochondria generate more than 90% of the cell endogenous oxidant species (Ames et al., 2002). Thus, alterations in the physiological function of mitochondria decrease their efficacy, leading to an increase in ROS production that triggers oxidative stress, which promotes disturbance of cell function, sensitizes cells to neurotoxic insults and leads to subsequent neuronal death (Johnstone et al., 2016).

In addition, the age-induced mitochondrial cascade of neurodegenerative events suggests that mitochondrial dysfunction accompanies ageing, and plays an important role in the AD pathogenesis (Bonda et al., 2010; Cavallucci et al., 2012). Indeed, it seems to be transversal to all AD stages and to worsen with disease progression (Maruszak and Zekanowski, 2011).

Interestingly, some studies in postmortem brains from AD patients, as well as in transgenic AD mice, and in cell lines expressing mutant APP and/or cells treated with Aβ, suggest that oxidative damage induced by Aβ is related with disturbed mitochondrial metabolism in AD development and progression (Manczak et al., 2006; Reddy and Beal, 2008; Spuch et al., 2012). The presence of Aβ within mitochondria provide a direct association between Aβ deposition and mitochondrial dysfunction due to the impair of mitochondrial respiratory function, increase of ROS generation, and change of mitochondrial membrane potentials, which are involved in neuronal metabolic dysfunction in AD (Cavallucci et al., 2012; Kim et al., 2015). It is also relevant to note that ROS produced by mitochondrial dysfunction seem to trigger amyloidogenic APP-processing, probably by BACE1-activated induction, leading to a vicious cycle that contributes to AD pathogenesis (Leuner et al., 2012). In addition, mitochondria-derived ROS can promote increased mitochondrial outer membrane permeability and cytochrome *c* (Cyt *c*) release into the cytosol, which can induce the apoptosome formation and the subsequent caspases activation (Cavallucci et al., 2012). Evidence also suggests that Aβ accumulation within neuronal cytoplasm impairs mitochondrial function, leading to energetic failure and generation of ROS, which determine functional neuronal impairment and subsequent neuronal death (Kidd, 2008).

Indeed, a large number of experimental and *postmortem* findings suggest a close relationship between AD and increased oxidative stress levels in the brain (Cervellati et al., 2016; Silva et al., 2012). Oxidative stress may result from an imbalance between

redox states, involving either excessive production of ROS, or dysfunction of the antioxidant system (Kim et al., 2015). Under healthy conditions, ROS are rapidly cleared to increasingly less reactive species by the enzymes catalase, glutathione peroxidase, and superoxide dismutase (SOD) 1 and 2 (Silva et al., 2012). However, when an excessive ROS production occurs, the endogenous reserves of antioxidants are not sufficient to counteract such generation, leading to oxidative cell damage (Silva et al., 2012).

Several studies suggest that levels of oxidative markers are directly correlated with the severity of cognitive impairment, as well as to symptomatic AD progression (Silva et al., 2012), which highlight that oxidative stress plays a key role in AD pathophysiology (Kim et al., 2015) and is an early manifestation of AD (Silva et al., 2012). In fact, oxidative markers generally precede all of those related with typical hallmarks of AD, like Aβ aggregation and NFTs formation, pointing their relevance as primary generators of AD pathology. Evidences demonstrate that Aβ secretion, as well as tau hyperphosphorylation and NFTs formation, initially occur as an antioxidant response to augmented ROS (Bonda et al., 2010). Thus, a chronic oxidative stress situation promotes Aβ production (Kim et al., 2015), which may induce the hallmark pathologies of AD, and intensifies the cascade of events that leads to neurodegeneration (Bonda et al., 2010). In addition, AD transgenic mouse models have shown that Aβ may be responsible for the production of hydrogen peroxide (H_2O_2), and peroxidation of lipids and proteins, implying that Aβ may enhance oxidative stress during AD progression (Kim et al., 2015).

The cause of AD is still unknown, but increasing evidences suggest that mitochondria play an important role in the oxidative stress process that underlies this disease, contributing to neurodegeneration (Bonda et al., 2010; Cassano et al., 2016). In addition, studies suggest that glial cells, like microglia and astrocytes, which release inflammatory cytokines and ROS, among other factors, contribute to neuronal damage (Lynch et al., 2010).

It is important to highlight that most likely all the hypotheses previously mentioned play some role in AD progression and that none of them are unique (Stone et al., 2015).

1.2.4. Neuroinflammation

Neuroinflammation is a feature frequently observed in *postmortem* brains of AD patients (Wu et al., 2015), particularly localized in brain areas exhibiting high pathological markers (Krause and Muller, 2010), thus corroborating its involvement in the disease (Janssen et al., 2015). The term neuroinflammation refers to an inflammation of the nervous tissue, as an intrinsic cellular response in the central nervous system (CNS) to

several stimuli, that may include infection, head injury, toxic metabolites, or autoimmunity, all of them contributing to AD progression (Pasqualetti et al., 2015; Peixoto et al., 2015) with degeneration of cholinergic neurons and memory impairment (Skok and Lykhmus, 2016). However, it is often accepted that neuroinflammation can have both beneficial and detrimental effects on neurons (Agostinho et al., 2010). While an acute neuroinflammatory response is beneficial to the CNS, since it promotes the repair of damaged tissue, a continued chronic neuroinflammation after an initial toxic stimulus may originate degenerative alterations in neurons leading to brain dysfunction (Eikelenboom et al., 2010; Lucas et al., 2006), and microglia senescence (Njie et al., 2012). In fact, studies in transgenic mouse models have showed that neuroinflammation has a diversified role in amyloid plaques and NFTs progression (Sudduth et al., 2013). Curiously, Colton and colleagues revealed that brains of mice with amyloid accumulation, and human AD tissue, express not only classical inflammatory genes, but also additional inflammatory markers (Colton et al., 2006), known to be important in damage repair (Sudduth et al., 2013). In addition, Sudduth and colleagues found remarkable heterogeneity of microglia polarization towards either the M1 or M2 inflammatory phenotype in early-stage of AD patients (Sudduth et al., 2013). Nevertheless, neuroinflammation is not exclusively associated with AD. In fact, healthy brains of aged individuals show inflammatory-related markers, which are even more increased during AD pathology. In addition, lower levels of inflammatory markers are found in individuals that exhibit Aβ and tau aggregates at levels identical to AD patients, but lacking a clinical presentation of dementia, reinforcing the idea that inflammation is required or associated to AD clinical symptoms (Krause and Muller, 2010).

Neuroinflammation seems to start early during AD (Parachikova et al., 2007; Xu et al., 2014), which is always accompanied by an exacerbated activation of many inflammatory pathways (Najem et al., 2014; Streit et al., 2014). Besides the abnormal accumulation of Aβ and tau protein (Echeverria et al., 2016), also includes the production of inflammatory mediators by resident CNS cells (pro-inflammatory cytokines, prostaglandins, free radicals and complement system), which in turn induce chemokines and adhesion molecules, recruit immune cells, and activate glial cells (Lucas et al., 2006). Indeed, gliosis, a prominent neuropathological feature, is observed in the brains of AD patients (Osborn et al., 2016), since an elevated number of activated astrocytes (astrogliosis) and microglia (microgliosis) are usually found in the proximity of neurons and amyloid plaques (Rubio-Perez and Morillas-Ruiz, 2012). Both astrocytes and microglia play several protective roles that contribute to neuronal homeostasis in the healthy brain. However, these activated glial cells may have both beneficial and detrimental effects. On the one hand, glial activation is an endogenous defensive

mechanism against plaque deposition, while on the other hand, the continued activation and associated inflammatory status may contribute to AD progression (Osborn et al., 2016). Thus, any change in their functions has repercussions on neuronal function leading consequently to cognitive impairments (Lynch et al., 2010; Osborn et al., 2016). In fact, astrocytes and microglia activation by Aβ deposition triggers neuroinflammatory processes by the production of several pro-inflammatory cytokines, among other signal molecules, promoting excitotoxicity and neurodegeneration (Lopategui Cabezas et al., 2012; Wyss-Coray, 2006; Wyss-Coray and Rogers, 2012), reason why they are part of the inflammatory components associated to neuroinflammation in AD (Rubio-Perez and Morillas-Ruiz, 2012).

Microglia were shown to alter their morphologic phenotype and produce several pro-inflammatory cytokines and mediators in response to Aβ (Manocha et al., 2015), which in turn activate astrocytes. In fact, astrocytes become highly reactive in response to any insult to the CNS, often stimulated by inflammatory cytokines (Eng et al., 2000). This astrogliosis is characterized by alterations in the cytoskeleton and morphological changes (hypertrophy) (Osborn et al., 2016), cell proliferation, and is associated with increased expression of glial fibrillary acidic protein (GFAP) (Eng et al., 2000). In addition, astrocytes appear to surround and isolate dying neurons, after neuronal damage (Lynch et al., 2010). Activated astrocytes in AD are part of the inflammatory process when they start to secrete the pro-inflammatory cytokines interleukin(IL)-1 and tumor necrosis factor(TNF)-α (Heneka et al., 2010). In addition, activated microglial cells become also a source of TNF-α, IL-1β, IL-1α, as well as superoxide, nitric oxide (NO), chemokines, and glutamate, which can promote neuronal death and contribute to onset and progression of AD (Block et al., 2007; Mizuno, 2012; Peixoto et al., 2015). Also, overexpression of astrocytic S100B induced by Aβo has a detrimental impact on neuronal survival and may further exacerbate the activation and migration of microglia (Bianchi et al., 2011; Mori et al., 2010). Furthermore, some of these inflammatory mediators associated to Aβ accumulation and tau phosphorylation, further activate glial cells, triggering a vicious neuroinflammatory cycle that contributes to the chronic inflammatory scenario observed in AD (Osborn et al., 2016), causing additional neuronal death.

Interestingly, it was suggested that age-dependent neuroinflammatory changes may play a significant role in the decreased neurogenesis and cognitive impairments in AD (Lynch et al., 2010; Varnum and Ikezu, 2012), while in late phases of AD is suggested that inflammation disappears (Wojtera et al., 2012). In fact, a recent study showed that AD has a transcriptional signature that is related to a metastable subproteome at risk of aggregation (Ciryam et al., 2016). Ciryam and colleagues found a global downregulation

in the expression of the genes that encode proteins that are metastable to aggregation, which is associated with the transcriptional response to AD (Ciryam et al., 2016). Moreover, many of the inflammatory processes observed in AD patients are similar to those present in the periphery, which are considered cytotoxic, and therefore have cytotoxic effects in the brain (Krause and Muller, 2010). In fact, AD patients who suffer a short-term peripheral infection manifest an abrupt cognitive decline, rarely achieving a full recovery, even after eliminating the infection (Perry et al., 2007). Indeed, treatment with nonsteroidal anti-inflammatory drugs (NSAIDs) have failed until now as protective against AD, unless the use is initiated in a very early stage of AD (Zou et al., 2016), to prevent disease progression (Krause and Muller, 2010; Varvel et al., 2009).

1.3. Diagnosis and treatment

The typical Alzheimer's symptom pattern begins with a subtle decline in short-term memory followed by a more general decline in overall cognitive abilities (Osborn et al., 2016), making AD one of the most significant healthcare problems nowadays (Bredesen, 2016). However, it is difficult to establish an accurate diagnosis of AD due to the frequent existence of co-morbidities in older people, which can also contribute to cognitive impairment (Alves et al., 2012). In addition, effective control of AD seems difficult to achieve because, in addition to having a relentless progression, AD diagnosis can only be performed on a stage already quite advanced of neurodegeneration (Kidd, 2008). In fact, the clinical diagnosis of AD can achieve an accuracy rate of 95% when realized by highly experienced clinicians (Alves et al., 2012), but a definitive diagnosis of AD is actually based on clinical and pathological hallmarks, as amyloid plaques and neurofibrillary tangles (Rubio-Perez and Morillas-Ruiz, 2012; Varvel et al., 2009), although it is now possible to identify the formation of amyloid plaques in patients using the Pittsburgh Compound-B (PiB)-PET imaging, as it will discussed below (Cohen et al., 2012). Since these hallmarks are definitively identified at autopsy, the Alzheimer's diagnosis is usually based on exclusion criteria (Kidd, 2008). So, there is a need for a non-invasive, cheap and validated test to diagnose early AD, as diagnosis may allow immediate treatment and service planning (Fletcher et al., 2013).

The most widely accepted diagnostic criteria for AD was developed by the National Institute of Neurological and Communicative Disorders and Stroke - Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) joint-working group in 1984, lately reviewed in 2011 by expert panels convened by the National Institute of Aging and the Alzheimer's Association (NIA/AA) (Ihara, 2016; McKhann et al., 2011). The diagnostic criteria established the definition of pre-clinical stages of AD (or pre-symptomatic phase) (Sperling et al., 2011), the diagnosis of a symptomatic or pre-

dementia phase (or MCI – mild cognitive impairment) (Albert et al., 2011), and the diagnosis of dementia phase (McKhann et al., 2011), and incorporated some biomarkers information used in research proposes to improve diagnosis (Alves et al., 2012; McKhann et al., 2011).

These biomarkers are physiological, biochemical, or anatomic parameters which can be objectively measured *in vivo* and reflect particular characteristics of disease-related pathophysiological processes, as well as normal biological processes, or responses to a therapeutic treatment (Jack et al., 2011; Jack and Holtzman, 2013). It is well known that both Aβ deposition and elevated phosphorylated tau are hallmarks of AD. As the deposition of Aβ seems to be more specific than changes in tau, the biomarkers were divided into two major categories. The first group includes biomarkers reflecting Aβ deposition in the brain, comprising both positive positron emission tomography (PET) amyloid imaging and low cerebrospinal fluid (CSF) $A\beta_{1-42}$. The second set is constituted by those related to neuronal degeneration or injury: elevated CFS tau, as well as both total and phosphorylated tau; decreased ^{18}fluorodeoxyglucose (FDG) uptake on PET in the temporo-parietal cortex; and atrophy on structural magnetic resonance imaging (MRI) in medial, basal and lateral temporal lobe, and medial and lateral parietal cortices (Jack et al., 2011; McKhann et al., 2011). Recently, it was reported that the most commonly used biomarker is MRI, followed by CSF, FDG, and amyloid-PET (Bocchetta et al., 2015), and the combination of amyloid and neuronal damaged biomarkers provides the most accurate prognosis of AD (Bocchetta et al., 2015; Vos et al., 2015). Beyond these, other promising biomarkers under research are the level of isoprostanes, which are markers of oxidative stress (Montine et al., 2011), and β-secretase (BACE1) in CFS (Zhong et al., 2007). Moreover, studies showed that a dysregulation of iron metabolism contribute to AD pathophysiology due to increased oxidative stress (Crespo et al., 2014; Guerreiro et al., 2015). Additionally, also *ApoE* gene, a well-known risk factor for AD, was identified as significantly related with this disease (Crespo et al., 2014).

Recently, it was suggested the usefulness of small extracellular vesicles, named exosomes, as biomarkers in the diagnosis of neurodegenerative diseases, including AD (Vella et al., 2016). It is possible that exosomes containing Aβ are released from neurons to clear and regulate increased levels of intracellular Aβ, which can be transferred into microglial cells and contribute to Aβ degradation (Yuyama et al., 2012), suggesting that exosomes may have a protective role in the brain of AD patients (An et al., 2013). Recent studies suggest that exosomes, which are generally isolated from extracellular fluid, can be extracted from brain tissue (Dinkins et al., 2014; Perez-Gonzalez et al., 2012). Indeed, exosomes can be isolated from extracellular fluids like CFS (Street et al., 2012; Vella et

al., 2008), blood (Cheng et al., 2014b) and urine (Cheng et al., 2013), suggesting its utility as peripheral non-invasive biomarker for AD (Vella et al., 2016). In fact, it was found CSF exosomes containing Aβ (Yuyama et al., 2014; Yuyama et al., 2015), and elevated total tau and $A\beta_{1-42}$ were detected in derived blood exosomes of AD patients (Fiandaca et al., 2014). Though plasma, serum and urine-based biomarkers have been studied, none of them showed a diagnostic accuracy as CSF biomarkers for AD (Anoop et al., 2010; Demarin et al., 2011). In addition, exosomes were found to be enriched with microRNA (miRNA) species (Gallo et al., 2012), which were differentially expressed in exosomes of AD patients and directly related with other methods of diagnosis (Cheng et al., 2014a), suggesting the use of exosomal miRNA as a complementary tool with other biomarkers to diagnose AD (Vella et al., 2016). Although it is believed that biomarkers will enable to improve diagnosis, and ultimately to provide an effective therapy (Jack et al., 2011), mainly at early AD stages, they are not yet ready for trials of clinical utility in primary care (Fletcher et al., 2013), and more studies are need to overcome their deficient standardization and their limited access (McKhann et al., 2011).

Beyond a deficient characterization of accurate biomarkers, the available pharmacological treatments are not cost-effective (Cacabelos et al., 2016). In addition, there are very few options in conventional medicine to treat AD (Kidd, 2008), although, for the past 20 years, several studies have been performed with more than 1000 different compounds in view of their use as potential candidate drugs for AD treatment. These candidate compounds can be classified according to their pharmacological properties and/or the AD-related pathway they target to avoid disease progression (Cacabelos et al., 2016). The Food and Drug Administration (FDA)-approved drugs for AD include acetylcholinesterase inhibitors (AChEIs), such as galantamine, donepezil and rivastigmine, which are prescribed to treat mild to moderate AD, and the *N*-methyl-D-aspartate (NMDA) antagonist memantine, which is prescribed to treat moderate to severe AD (Alves et al., 2012; Raina et al., 2008). AChEIs inhibit the degradation of acetylcholine, the major neurotransmitter related with attention and memory, slowing the rate of cognitive decline, while NMDA antagonist prevent neurotoxicity in the brain by reducing excessive glutamate activation (Nelson and Tabet, 2015; Parsons et al., 2013). However, although these pharmaceutical drugs show to delay some symptom evolution, they cannot stop AD progression (Echeverria et al., 2016; Khairallah and Kassem, 2011). Lamentably, though these limitations these drugs are the only available therapies, because all other new drugs tested until now failed to improve cognitive abilities in phase 3 clinical trials (Echeverria et al., 2016). Thus, several studies are being made in an attempt to develop new effective treatments for AD.

A candidate strategy to AD treatment are novel cholinergic agents including direct and allosteric muscarinic acetylcholine receptor agonists and also agonists of some subtypes of nicotinic receptors (Alves et al., 2012), the latter having been reported to be decreased in AD brains. Indeed, modulators of α7nAChRs, such as nicotine and some of its derivatives, can decrease the abnormal activation of microglia and diminish neuroinflammation, abnormal protein aggregation and synaptic dysfunction, because of their anti-inflammatory, anti-apoptotic, anti-protein aggregation and pro-cognitive effects. Nonetheless, the fact that the α7nAChRs are rapidly desensitized, they regarded as a constraining factor to their potential therapeutic use (Echeverria et al., 2016).

The suggested correlation between neuroinflammation and AD (Janssen et al., 2015), lead to several epidemiological studies of NSAIDs showing their anti-inflammatory effect in AD (McGeer et al., 2006). However, so far, these drugs revealed limited benefits and to be an ineffective treatment in clinical trials (Echeverria et al., 2016; McGeer et al., 2006). Indeed, treatment with NSAIDs has failed until now as protective against AD, unless the use is initiated as early as possible to prevent disease progression (Krause and Muller, 2010; Varvel et al., 2009). This probably occurs due to the previous deterioration in cognitive function in AD patients prior to medication, or to several pathways other than cyclooxygenase-dependent activity target by NSAIDs (Ryu et al., 2015). A recent analysis of clinical trials studying the effectiveness of the NSAIDs diclofenac/misoprostol, nimesulide, naproxen, rofecoxib/ibuprofen, indomethacin, tarenflurbil, and celecoxib, showed no clinical significance of the treatment with these drugs when compared with placebo in AD progression, which has been attributed to the dose and its administration at advanced stages of the disease (Miguel-Alvarez et al., 2015).

Also immunotherapy has been suggested for AD treatment (Villoslada et al., 2008), as a therapeutic approach to clear Aβ aggregates, and/or tau hyperphosphorylated in AD brains, by the use of antibodies against these proteins. In fact, active immunization with Aβ vaccines reduced amyloid aggregates, as well as memory and learning deficits in animal models. However, it can induce undesired adverse autoimmune responses that can be fatal (Alves et al., 2012; Echeverria et al., 2016). New attempts have been made to find safe approaches such as the passive immunizations with antibodies targeting different regions of the Aβ peptide, which are actually in various stages of clinical trials. However, some of the monoclonal antibodies used may damage the cerebral vasculature already affected by Aβ accumulation (Echeverria et al., 2016). In addition, also immunization to target tau pathology allowed tau clearance and improved cognitive deficits in a tau transgenic model, but again it can

be deleterious if targeting non-phosphorylated tau since it is a normal neuronal component (Rosenmann et al., 2006; Troquier et al., 2012).

Some drugs designed to reduce Aβ production by inhibiting β- or γ-secretase, blocking calcium channel, or stimulating α-secretase, as well as preventing Aβ aggregation, have also been tested, but they did not show global clinical benefits (Alves et al., 2012). Although most of the treatments have Aβ as the main target, drugs aiming at inhibiting tau hyperphosphorylation, tau oligomerization or at promoting hyperphosphorylated tau degradation, were investigated (Jiang et al., 2012b). Recently, oral administration of the naturally occurring monoterpene linalool to the triple transgenic model of AD (3xTg-AD) mice reversed the histopathological hallmarks of AD and restored cognitive functions via an anti-inflammatory effect, pointing to a potential AD prevention candidate for preclinical trials (Sabogal-Guaqueta et al., 2016). In addition, light in the near infrared range is emerging as a safe and effective therapy with the ability to stop neuronal death, which is a feature of AD (Johnstone et al., 2016). Various other strategies are under research, such as mitochondria-targeted antioxidants to inhibit mitochondrial dysfunction and oxidative stress (Isaev et al., 2015), pharmacological modulation of the receptor CXCR2 to inhibit receptor-mediated inflammatory reactivity (Ryu et al., 2015), inhibitors of specific nuclear factor of activated T cells (NFAT) expressed by microglial cells to attenuate microgliosis and Aβ plaque deposition (Rojanathammanee et al., 2015), and tyrosine kinase inhibition to reduce microgliosis (Dhawan and Combs, 2012).

Evidence so far indicates that therapies targeting a single hallmark of AD show limited efficacy in reducing disease progression. Probably, drugs alone or in combination, that are able to target more than one aspect of AD pathology, including the cholinergic deficit, neuroinflammation, and neuronal loss, as well as Aβ and tau abnormal aggregation, may have more chance of success in decreasing the negative side effects of neuroinflammation while maintaining its beneficial effects (Echeverria et al., 2016).

2. Microglia involvement in AD

Increasing evidences suggest the influence of microglial activation in AD pathology, being Aβ hypothetically responsible for this activation that leads to a pro-inflammatory response by microglia, thereby contributing to disease progression (Dhawan and Combs, 2012; Rojanathammanee et al., 2015).

2.1. Microglia origin and functions

Microglia are one of the three glial cells (together with oligodendrocytes and astrocytes) found in the CNS (Krause and Muller, 2010), and have been first mentioned by Pio del Rio-Hortega (del Rio-Hortega, 1993), constituting around 10% of the total brain cell population (Echeverria et al., 2016; Rubio-Perez and Morillas-Ruiz, 2012). Although microglia cells are distributed ubiquitously throughout the normal CNS, in both grey and white matter, their density is higher in the hippocampus, substancia nigra, olfactory telencephalon, and basal ganglia (Lawson et al., 1990; Tambuyzer et al., 2009). Despite some controversy about the origin of microglial cells, the general consensus is that microglia have hematopoietic origin, being derived from myeloid precursor cells from yolk sac that migrate into the brain during embryogenesis (Ginhoux et al., 2013; Greter and Merad, 2013).

In the normal brain, and under normal conditions, microglial cells display a highly branched and ramified morphology, with a small body and long, thin processes with dynamic protrusions to constantly survey the surrounding environment (Kettenmann et al., 2013; Nimmerjahn et al., 2005). Each cell seems to occupy a defined territory, not overlapping with neighbouring microglial cells (Kettenmann et al., 2011). In addition, although microglia are regarded as long-lived cells, microglia homeostasis may be maintained by local self-renewal under physiological conditions (Ajami et al., 2007; Greter and Merad, 2013).

Microglia exhibit some properties of macrophages as part of the mononuclear phagocyte lineage (Vilhardt, 2005), and, in fact, they are considered the resident immunocompetent and phagocytic cells in the CNS, as they act as the innate immune brain system (Aloisi, 2001; Streit, 2002). However, more recent studies show that, at resting conditions, microglia differ from macrophages since they express much lower levels of cluster of differentiation protein(CD)45, being the only available way to distinguish them phenotypically (Greter and Merad, 2013). Healthy brain microglia act as sentinels and change their phenotype in response to any homeostatic disturbance, such as pathogenic invasion or tissue damage (Davalos et al., 2005; Nimmerjahn et al., 2005), migrating to the local of injury (Rubio-Perez and Morillas-Ruiz, 2012). Some of the markers commonly used to evaluate microglial reactivity are the β-integrin CD11b, also known as complement receptor(CR)-3, and the ionized calcium-binding adaptor molecule 1 (Iba1) protein, which are upregulated in activated microglia (Ito et al., 2001; Kim and de Vellis, 2005; Roy et al., 2006).

Similar to macrophages, in order to detect potential insults that disturb CNS homeostasis, microglia express several different surface receptors, including major histocompatibility complex class II (MHC class II) antigens and T-cell costimulatory

molecules, such as CD40, CD80 and CD86 (Ransohoff and Perry, 2009). In addition, microglia can also express neurotransmitter receptors and pattern recognition receptors (PRRs), such as toll-like receptors (TLRs), or nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), that recognize danger-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) (Greter and Merad, 2013; Heneka et al., 2015; Kettenmann et al., 2011). After recognition of a DAMP or a PAMP, the microglial activation occurs intending to remove the detected abnormality or pathological change (Heneka et al., 2015), which leads to a change of their ramified morphology to an amoeboid shape (Kettenmann et al., 2011). Indeed, microglia are constantly surveilling, with small cell bodies and long, thin processes, changing to an activated state upon a stress-stimulus, with a phagocytic morphology (amoeboid) and shifting from long to short processes presentation (Zlokovic, 2008). By phagocytosis, microglia can remove cellular debris, damaged cells, unwanted protein aggregates, or pathogens, from CNS (Becher et al., 2000; von Bernhardi, 2010), and support tissue repair and remodelling (Ginhoux et al., 2013). However, TLR-induced microglia activation promotes the upregulation of MHC class II (Olson and Miller, 2004), having these activated microglia an important role in onset and progression of neurodegenerative diseases, as AD, by the release of potentially cytotoxic molecules, like pro-inflammatory cytokines, ROS and inducible nitric oxide synthase (iNOS) (Block et al., 2007; Greter and Merad, 2013; Kettenmann et al., 2013). These features suggest that their phagocytic function can be beneficial while their inflammation-related functions may be detrimental (Krause and Muller, 2010).

In addition to their immune functions, emerging results demonstrated the role of microglia in controlling neuronal proliferation and differentiation, as well as in formation of synaptic connections (Graeber, 2010; Hughes, 2012), as well as in modification and removal of synaptic structures (Tremblay et al., 2010). Thus, microglia, besides the immune role, have distinct gene expression signatures highlighting functions exclusively adapted to the CNS, such as synaptic modulation and neurotrophic support (Wes et al., 2016), promoting the defence and the maintenance of the CNS (Ginhoux et al., 2013).

2.2. Microglia activation in AD

Microglia can present at least five clearly recognizable stages in the adult CNS: the surveilling or ramified microglia, which move dynamically and are distributed ubiquitously throughout the normal and nonpathological CNS; the activated or reactive microglia, which appear in pathological conditions, although not always phagocytic but hypertrophic with wide processes; the phagocytic microglia, which occur as amoeboid brain macrophages; the rod-shaped microglia with a narrow cell boby and few planar

processes; and the dystrophic microglia, which are senescent cells with spheroidal or fragmented processes (Bachstetter et al., 2015). In fact, microglia are active sensors in the brain that rapidly switch to an adequate functional activity state in response to a variety of stimuli in order to restore microenvironment homeostasis **(Figure 1.4)**. This microglial activation depends on the stimuli to which the cells are exposed and can explain why engagement of microglia can be either neuroprotective or neurotoxic, resulting in a delay or worsening of disease progression (Hanisch and Kettenmann, 2007; Schwartz et al., 2006). In what concerns AD, it has been indicated that microglia may be activated by oligomeric and fibrillar species of Aβ and by molecules derived from degenerated neurons (Mizuno, 2012).

The terms resting or quiescent to define the behaviour of microglia in the healthy brain do not reflect what really occurs. It suggests a kind of inactivity that is not compatible with the constant movement of ramified microglial processes used to dynamically monitor the microenvironment, always in a surveilling state to maintain homeostasis (Gomez-Nicola and Perry, 2015; Kettenmann et al., 2011; Nimmerjahn et al., 2005). In contrast, reactive or activated microglia can acquire diverse and complex altered morphologies/phenotypes, including hypertrophic cell morphology with enlarged processes or amoeboid morphology, enabling their participation in the cytotoxic response, immune regulation and damage resolution (Chhor et al., 2013; Gemma and Bachstetter, 2013). Interestingly, a recent study showed a high heterogeneity in microglia morphology in the human brain, which is usually underestimated since this diversity is scarcely seen in animal models (Bachstetter et al., 2015). In addition, a large part of ramified microglia observed in the healthy brains, become dystrophic with AD progression, although microglial morphology diversity still remains (Bachstetter et al., 2015).

Regarding activation, and depending on the stimulus and the level of activation, microglia were categorized into different phenotypes **(Figure 1.4)**, being the M1 and M2 subtypes the ones most often appointed (Brites and Vaz, 2014). The M1 phenotype, also known as classically activated, is induced by pro-inflammatory mediators, such as lipopolysaccharide (LPS) and interferon(IFN)-γ (Czeh et al., 2011). Although initially intending to protect and repair the CNS, the cell also display cytotoxic properties (Cherry et al., 2014; Chhor et al., 2013), by overproduction of mediators to kill the pathogenic agent, namely pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6 and IL-12, and several chemokines, proteases, redox proteins, ROS, and iNOS, that uses arginine to produce NO (Boche et al., 2013; Cherry et al., 2014; Colton, 2009; Kraft and Harry, 2011). The M2 phenotype, or the alternatively activated state, is related with inhibition of inflammation and homeostasis recovery (Brites and Fernandes, 2015), by production of

anti-inflammatory cytokines, like IL-4, IL-13, IL-10 and transforming growth factor(TGF)-β (Colton, 2009). In addition, the M2 phenotype may be subdivided into different subtypes, including M2a, with an alternate activation associated with the dampen of inflammation by production of anti-inflammatory cytokines and neurotrophic factors (Chhor et al., 2013; Colton, 2009; Kraft and Harry, 2011); M2b is considered to be both pro- and anti-inflammatory, i.e., to show combined M1/M2a subtypes (Brites et al., 2015); and M2c, or acquired-deactivating phenotype, related with phagocytosis and suppression of the innate immune system (Brites and Vaz, 2014; Chhor et al., 2013). This phenotype downregulates innate immune responses and show a distinct gene profile. Acquired deactivation is distinguished from alternative activation by the induction agents. While alternative activation is induced by IL-4 or IL-13, acquired deactivation is produced by inducing agents such as TGF-β, IL-10 and apoptotic cells, leading to the suppression of MHC class II antigens, inhibition of the production of pro-inflammatory cytokines, increase of the expression of scavenger receptors (SRs), increase of neural growth factor (NGF) levels, as well as of IL-10 and TGF-β production, both anti-inflammatory cytokines (Colton, 2009; De Simone et al., 2004; Minghetti et al., 2005; Minghetti and Pocchiari, 2007). Nonetheless, one should be careful in considering that the M2 phenotype is always beneficial, since it was pointed as corresponding to a deactivated, irresponsive population (Chakrabarty et al., 2012), and both M2a and M2c microglia were shown to be implicated in AD progression in the APP/PS1 transgenic mice (Weekman et al., 2014). Really, it is believed to be too simplistic to consider microglial cells either as beneficial or detrimental, since microglia are able to switch between these phenotypes and may occur in several intermediate states (Brites and Fernandes, 2015). Other M2 markers often used are the enzyme arginase 1 which competes with iNOS for substrates, the chitinase 3-like protein 3 (Ym1), a heparin-binding lectin, which prevent degradation of extracellular matrix compounds, and the protein found in inflammatory zone 1 (FIZZ1) which promotes extracellular matrix deposition (Cherry et al., 2014; Raes et al., 2002). In addition, in the context of immunoregulatory characteristics, M1 microglia phenotype express higher levels of MHC class II, CD80, CD86 and chemokine receptor CCR7, while M2 microglia is associated with upregulated levels of CD209 (DC-SIGN) (Cherry et al., 2014; Durafourt et al., 2012).

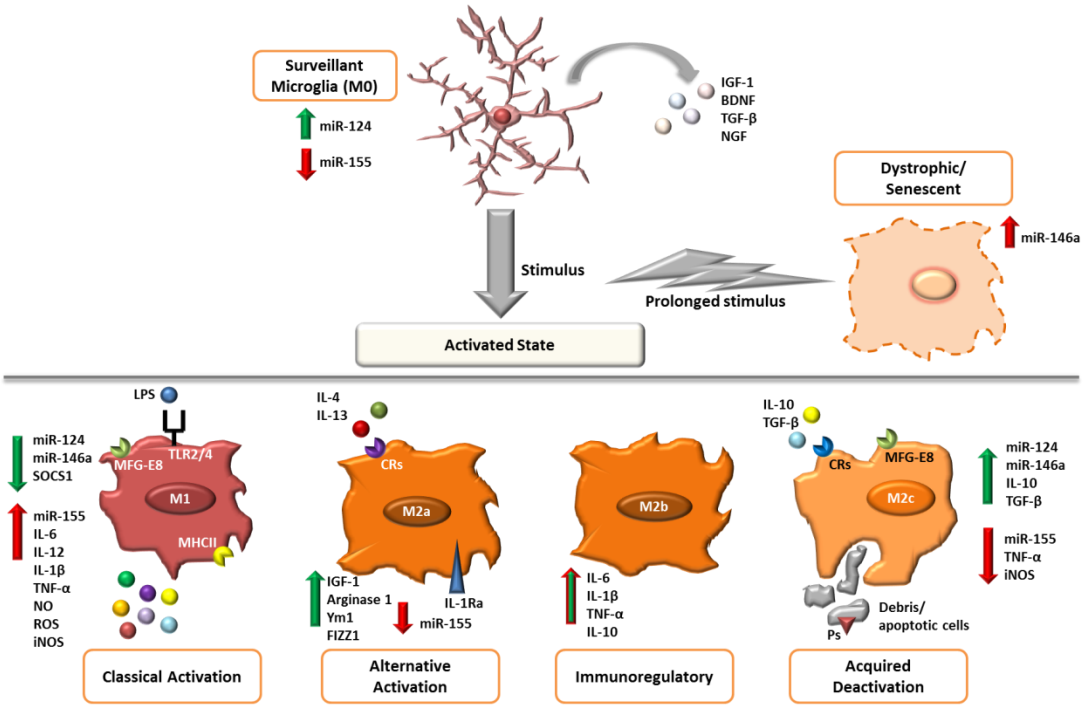

Figure 1. 4 – Microglia activation states/phenotypes. In the healthy central nervous system, microglia present a highly ramified morphology with long and thin processes, which dynamically monitor brain parenchyma to maintain the homeostasis. These microglial cells are commonly designated as surveillant microglia, or M0 phenotype, which produce several beneficial substances, such as insulin-like growth factor(IGF)-1, transforming growth factor (TGF)-β, brain-derived neurotrophic factor (BDNF), and nerve growth factor (NGF), being characterized by low expression levels of microRNA (miR)-155 and relatively high expression of miR-124. Depending on the stimulus, surveillant microglia may react and show different activation profiles with distinct morphologies, from hypertrophic with enlarged processes to amoeboid shape. The pro-inflammatory lipopolysaccharide (LPS) induces a classical activation of microglia (M1), mainly through toll-like receptors (TLRs)2 and 4, resulting in a pro-inflammatory profile characterized by an increased production of interleukin (IL)-6, IL-12, IL-1β, tumour necrosis factor (TNF)-α, nitric oxide (NO), reactive oxygen species (ROS), inducible nitric oxide synthase (iNOS), and major histocompatibility complex class II (MHC class II) antigens. Besides their supportive role, the overproduction of these mediators is commonly associated to neurotoxicity. Beyond the downregulation of miR-124 and miR-146a, the miR-155 upregulation is thought to have a pivotal role in the establishment of the M1 phenotype, since this miRNA directly targets anti-inflammatory molecules, such as suppressor of cytokine signalling (SOCS) 1. In contrast, alternative activation (M2a) and acquired deactivation (M2c) are both associated with an anti-inflammatory and neuroprotective phenotype, being the first characterized by the upregulation of IGF-1, arginase 1, chitinase 3-like protein 3 (Ym1) and IL-1 receptor antagonist (IL-1Ra), and related with tissue repair, while the second one by releasing IL-10, is more associated with the suppression of the immune system and phagocytosis of cellular debris. These last phenotypes also show a downregulation of the pro-inflammatory miR-155 expression, and an upregulation of miR-124 and miR-146a. In addition, phagocytosis can also be mediated by the release of milk factor globule-8 (MFG-E8) from microglia M1 and M2c phenotypes which recognizes phosphatidylserine (Ps) in apoptotic cells. The M2b immunoregulatory phenotype has as reliable markers IL-10, IL-6, IL-1β and TNF-α, being considered a combination between anti- and pro-inflammatory phenotypes. To note that after a prolonged stimulus, microglia may become dystrophic or senescent, a condition where an increased expression of miR-146a is observed.

In regard to M1 and M2 polarization, some small endogenous RNA molecules, known as microRNAs (miRNAs), have been described as mediators of inflammation, either as modulators of pro-inflammatory response or related with suppression of the pro-inflammatory microglial behaviour (Freilich et al., 2013; Su et al., 2015). These miRNAs act by modulation of complementary messenger RNA (mRNA) molecules or by inhibiting their translation through binding to the 3' untranslated region (3'UTR) (Cardoso et al., 2012). The complementary between the nucleotides 2-8 on the 5' region of the miRNA and the mRNA is responsible for this binding, allowing the recognition of different mRNA targets simultaneously by a single miRNA molecule (Bartel, 2009), giving them the ability to modulate a great number of proteins (Su et al., 2015), and making them a set of molecules with potential to be used in diagnostics and therapy (Guedes et al., 2014). The miRNA mediated regulation of gene expression is reached either by translation repression or degradation of the mRNA target molecule (Macfarlane and Murphy, 2010), and has been related with inflammation and diverse essential processes, such as apoptosis, proliferation, differentiation, development and angiogenesis (Guedes et al., 2013).

Several miRNAs associated with innate immunity and neuroinflammation have been shown to be dysregulated in AD (Guedes et al., 2014). MiR-155 is a pro-inflammatory miRNA, playing a significant role in the modulation of the innate immune responses by regulation of chemokine and cytokine production (Guedes et al., 2014). In this respect, it has been shown that miR-155 is upregulated in microglial cells after a pro-inflammatory stimulus (Lu et al., 2011), inducing the M1 phenotype **(Figure 1.4)** (Ponomarev et al., 2013) and the overexpression of several inflammatory mediators by targeting the anti-inflammatory-related protein suppressor of cytokine signalling(SOCS)1 (Cardoso et al., 2012). Furthermore, it has also been demonstrated that miR-155 is able to target M2-associated genes, including CCAAT/enhancer-binding protein (CEBP)-β, a transcription factor crucial for IL-10, arginase 1, and CD206 expression, as well as the one encoding SMAD2, a protein involved in the TGF-β pathway (Guedes et al., 2013). In addition, miR-155 expression has also been found increased in the 3xTg-AD mice model (Guedes et al., 2014), while in the SOD1 mice the genetic ablation of miR-155 restored the microglia phagocytic ability (Butovsky et al., 2015). Also miR-124 , firstly identified as a mouse brain-specific miRNA and the most predominant in the brain (Lagos-Quintana et al., 2002), has been reported to promote microglial M2 phenotype **(Figure 1.4)**, including a reduction of M1-related markers expression, like IL-6, TNF-α, and iNOS, and an increasing of markers related with the M2 phenotype, such as TGF-β,

arginase 1, and FIZZ1 (Ponomarev et al., 2011). Indeed, knock-down of miR-124 in microglia induced a pro-inflammatory activation *in vitro* and *in vivo* (Bird, 2011; Ponomarev et al., 2011). Additionally, while an overexpression of miR-124 has been found in nonactivated microglia cells ($CD45^{low}$/MHC class II^{low}), a marked decrease occurred following treatment with IFN-γ and granulocyte-macrophage colony-stimulating factor (GM-CSF), which induce the expression of CD45 and MHC class II and promote the M1 phenotype (Ponomarev et al., 2011). Interestingly, cultured microglia have shown decreased expression of miR-124 when exposed to LPS, or when treated with IL-4, a cytokine that promote alternative activation (Freilich et al., 2013), thus being its expression supressed by both types of activation signals. More recently, it has also been suggested that miR-124 is involved in the surveillance state of microglia by triggering transcriptor factor CEBP-α (Ponomarev et al., 2011). Curiously, miR-124 expression occurs abundantly in microglial cells but not in other tissue macrophages (Liu and Abraham, 2013). In addition, miR-146a is another well studied regulator of innate immune response, acting as a negative feedback promotor of pro-inflammatory responses (Su et al., 2015), by targeting several key inflammatory mediators (Cui et al., 2010). Indeed, a pro-inflammatory induction through nuclear factor-kappa B (NF-κB) transcriptional pathway promotes an increased expression of miR-146a, which in turn inhibits NF-κB transcriptional activity (Li et al., 2011). It was also suggested that a reduced expression of miR-146a can promote hyperactivation or prolonged inflammation (Liu and Abraham, 2013). In fact, in Aβ and TNF-α stressed human microglial cells, the expression of miR-146a was inversely correlated with the level of inflammation-associated proteins (Li et al., 2011). Additionally, miR-146a has been directly related with AD (Alexandrov et al., 2014; Lukiw et al., 2008), as well as implicated in age-related dysfunction of macrophages (Jiang et al., 2012a), as depicted in **Figure 1.4**. Interestingly, studies in PS2 dysfunction, originated by aging or mutations, suggested that it may promote neurodegeneration by inducing microglial pro-inflammatory responses through misregulation of miR-146a (Jayadev et al., 2013).

Ultimately, evidence demonstrate that miRNAs can play crucial regulatory roles when acting together, leading to alteration of neuroimmune functions (Su et al., 2015). In fact, miR-155 and miR-146a are often associated to act together in modulation of several stages of the innate immune response during inflammation and infection (Elton et al., 2013; O'Connell et al., 2010), namely in the microglial inflammatory profile (Su et al., 2015). While miR-155 usually induces microglia-mediated pro-inflammatory responses, miR-146a supresses inflammation by inhibition of NF-κB transcriptional activity (Su et al., 2015). Additionally, miR-146a has also been demonstrated to negatively modulate expression of pro-inflammatory cytokines, such as TNF-α and IL-6,

in contrast with miR-155 which increases pro-inflammatory responses, by inhibiting expression of anti-inflammatory mediators, and may play a role of negative feedback regulator of miR-146a (Su et al., 2015).

Therefore, microglia are seen as the guardian of the CNS providing the first line of defence whenever it occurs any injury or disease. In this concern, microglia perform various functions varying from immunological surveillance to neuroprotection or neurotoxicity, including migration to the site of damage, proliferation, morphological changes, phagocytosis of cellular debris, synaptic modulation, and production of inflammatory mediators.

2.2.1. Microglia-Aβ peptide interaction

Several studies have revealed the accumulation of phenotypically activated microglia surrounding amyloid plaques (Cameron and Landreth, 2010; Floden and Combs, 2011; Lue et al., 2010) in AD patients (McGeer et al., 1987), as well as in AD mouse models (Frautschy et al., 1998; Yan et al., 2009), being considered a hallmark of AD pathology (Doi et al., 2009). Indeed, in age-matched human controls, which do not show Aβ deposits, microglial agglomeration does not occur (Streit, 2002). This microglial accumulation can be explained by chemotactic signalling mediated by Aβ, or by other molecules also related with plaques, like complement factors or chemokines (Lue et al., 2001; Sokolowski and Mandell, 2011). In fact, it was observed by *in vivo* imaging techniques that microglia migrate and accumulate at newly formed amyloid plaques (Meyer-Luehmann et al., 2008), being their number proportional to the size of plaques and their presence indicated as preventing plaque expansion, suggesting that plaque-associated microglia can manage plaque dynamics (Bolmont et al., 2008). In addition, microglial activation depend on the amyloid load (Rubio-Perez and Morillas-Ruiz, 2012), and has been considered an early event in the pathogenesis of AD (Cagnin et al., 2001). However, microglia undergo an age-associated decrease in their ability to interact with Aβ aggregates (Floden and Combs, 2011).

In AD, Aβ peptides, either as monomers, oligomers, and/or fibers, which are compounds of amyloid plaques (Mizuno, 2012), constitute the most likely immune stimulus (Colton, 2009), as it induces a high expression of MHC class II surface molecules (McGeer et al., 1988). In fact, it was shown that Aβ induces activation of microglia (El Khoury and Luster, 2008; Solito and Sastre, 2012). The microglia-Aβ interaction depends on the physical and biochemical properties of Aβ (Lue et al., 2010), once it differs depending on whether it will be Aβf and Aβo (Mizuno, 2012). Both Aβf and Aβo are able to bind and induce activation of microglia through several receptors **(Figure**

1.5), including different PRRs (Salminen et al., 2009). Indeed, Aβ may act as a PAMP molecule and interacts with TLRs expressed by microglial cells (Cameron and Landreth, 2010; Lotz et al., 2005; Reed-Geaghan et al., 2009), among which TLR2 and TLR4 were shown to be involved in Aβ-induced microglial activation (Liu et al., 2012b; Lue et al., 2010; Mizuno, 2012; Salminen et al., 2009; Stewart et al., 2010). Indeed, it was observed that TLR2 mediates Aβ peptide-induced activation of microglia, promoting the expression of iNOS, pro-inflammatory cytokines, such as TNF-α, IL-1β and IL-6, and integrin markers, including CD11b (Jana et al., 2008). Moreover, several *in vitro* and *in vivo* studies have shown that TLR4 mediates the neurotoxicity induced by microglia (Lehnardt et al., 2003; Walter et al., 2007). Beyond TLRs, other receptors were demonstrated to be involved in Aβ-induced microglial activation. These include RAGE receptors (Meraz-Rios et al., 2013), TLR coreceptor CD14 (Landreth and Reed-Geaghan, 2009; Liu et al., 2005), other scavenger receptors, such as CD36 and CD47 (Bamberger et al., 2003; Salminen et al., 2009), and purinergic P2X7 receptor (P2X7R) (Sanz et al., 2009). The microglial activation induced by Aβ through these receptors promotes microglia migration and causes inflammatory responses against Aβ aggregates, inducing the production of NO, ROS and pro-inflammatory cytokines (Cameron and Landreth, 2010; Heneka et al., 2015; Mantovani et al., 2004; Mizuno, 2012). The activation of signalling cascades **(Figure 1.5)** mediated by the NF-κB-dependent pathway (Combs et al., 2001) may directly originate neuronal damage (Meraz-Rios et al., 2013).

TLRs, and the coreceptor CD14, may likewise enhance Aβ phagocytic uptake by microglia (Doi et al., 2009; Liu et al., 2005), being both TLR2 and TLR4 necessary for Aβf-stimulated phagocytosis (Reed-Geaghan et al., 2009). Thus, activated microglia can be beneficial, since they can reduce Aβ accumulation through increased phagocytosis, clearance and degradation (Cameron and Landreth, 2010; Morgan, 2009; Solito and Sastre, 2012), though the progressive accumulation of Aβ in AD disease may promote a chronic stimulus to microglia (Krause and Muller, 2010). Microglia also internalize soluble Aβ from the extracellular milieu through a macropinocytic mechanism that is distinct from that of phagocytosis (Mandrekar et al., 2009).

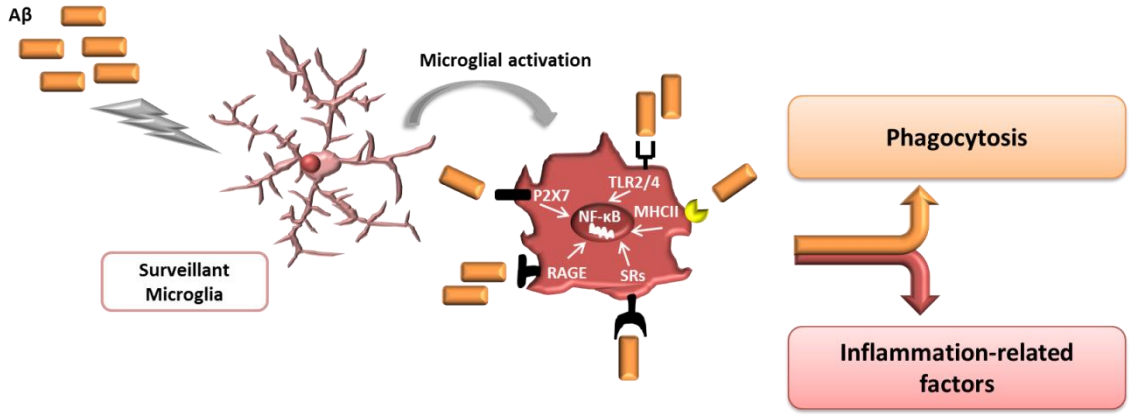

Figure 1. 5 – Interaction between microglial receptors and amyloid-β (Aβ) peptide. Microglia express several surface receptors and may sense signals from the surrounding environment and respond to different stimuli. Amyloid-β (Aβ) is able to interact with several of these receptors, promoting microglial activation. Among the receptors involved in Aβ binding are: toll-like receptors (TLRs), such as TLR2 and TLR4; scavenger receptors (SRs), like CD36, CD47 and CD14; receptor for advanced glycation endproducts (RAGE); purinergic P2X7 receptor (P2X7R); and major histocompatibility complex class II (MHC class II) molecules. The binding of Aβ to microglial receptors trigger a pro-inflammatory classical activation state in microglia, with the release of pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α, and interleukin (IL)-1β and IL-6, probably via activation of the nuclear factor-kappaB (NF-κB) signalling pathway, although it may also induce phagocytosis.

It should be noted that, even though both Aβf and Aβo are able to promote the classical microglial activation, they display different activation profiles (Parvathy et al., 2009; Sondag et al., 2009), having Aβo revealed to be a stronger inducer of M1 phenotype than Aβf in the same conditions (Maezawa et al., 2011). Actually, Aβo at low nanomolar concentrations (5-50 nM), although not neurotoxic, induces indirect microglia-mediated neurotoxicity by increasing the production of NO (Maezawa et al., 2011). Thus, Aβo may more potently cause neuronal damage indirectly by activating microglia in AD than plaque-associated microglia, playing a more significant role in the cognitive decline of AD patients (Maezawa et al., 2011). However, Aβo concentrations ranging from 100 nM to 10 μM were found to cause neuronal death (Nicholson and Ferreira, 2009), and reduce survival of mature oligodendrocytes (Horiuchi et al., 2010). Interestingly, has also been shown that nanomolar concentrations of Aβ promote microglia phagocytosis of viable neurons and synapses in culture, which may modulate neuronal loss and AD progression (Neher et al., 2012; Neniskyte et al., 2011). Taking these findings in consideration, it is likely that Aβo can reach the low nanomolar concentrations required for microglia activation in the very early stages of AD development and progressively accumulate into higher and more neurotoxic concentrations priginating specific AD symptoms (Hardy and Selkoe, 2002).

2.2.2. Microglia release of inflammation-related factors

In the AD brain, interaction between microglia and Aβ induces the phenotypic activation of microglia and release of pro-inflammatory mediators (Cameron and Landreth, 2010). Microglia, along with astrocytes, are the major source of cytokines in AD, which contribute to many inflammatory-related events, including pro- and anti-inflammatory processes, chemoattraction, and response of microglia to Aβ. Thus, microglial activation can be both characterized and modulated by cytokines (Heneka et al., 2015). Indeed, several works, either *in vitro* or animals models, demonstrate that increases in Aβ concentration are related with enhanced release of pro-inflammatory cytokines by Aβ-induced microglia, including TNF-α, IL-6, IL-1β, and IL-1α (Lue et al., 2001; Patel et al., 2005; Rubio-Perez and Morillas-Ruiz, 2012), which eventually could promote neuronal death (Akiyama et al., 2000). These cytokines may further induce cytokine production by acting in an autocrine manner or by activating astrocytes (Rubio-Perez and Morillas-Ruiz, 2012). In fact, inflammatory cytokines have been shown to be increased in vulnerable brain regions in AD, and were found to be associated with amyloid plaques (Agostinho et al., 2010; Rubio-Perez and Morillas-Ruiz, 2012).

Furthermore, Aβ-induced microglia, together with astrocytes, are the main producer of chemokines in the CNS (Meraz-Rios et al., 2013). Chemokines, such as monocyte chemotactic protein-1 (MCP-1 or CCL2) and macrophage inflammatory protein-1α (MIP-1α or CCL3), as well as chemokine receptors CCR3 and CCR5, in reactive microglia surrounding senile plaques of AD patients were already identified (El Khoury et al., 2003; Meraz-Rios et al., 2013; Xia et al., 1998). Thus, it is consensual that such chemokines in the CNS are able to promote recruitment of microglial cells and astrocytes to the site of Aβ aggregation and to the area of neuroinflammation, expanding the extent of local inflammation and leading to chronic production of chemokines, which contribute to AD progression (Meraz-Rios et al., 2013; Rubio-Perez and Morillas-Ruiz, 2012).

Several studies have shown interaction between Aβ and the production of other inflammatory-related molecules, like ROS and reactive nitrogen species (RNS), by microglial cells (Meda et al., 1995). The ROS generation is also frequently related with production of pro-inflammatory factors by activated microglial cells to eliminate pathogens and to mediate destruction of cellular debris, but a high production of ROS by prolonged microglial activation can aggravate inflammation, entering in a vicious inflammatory cycle, which may induce neuronal death (Kraft and Harry, 2011; Pasqualetti et al., 2015). As ROS, NO can be either detrimental to neurons and be implicated in the onset and progression of multiple brain insults, including AD (Guix et al., 2005; Pasqualetti et al., 2015), or induce neuroprotective signaling events (Kraft and Harry,

2011). Indeed, under normal conditions, NO is synthesized in the microvasculature and regulates β-secretase activity which participates in APP processing (Austin et al., 2010). Other studies have shown that NO release by microglia can result from several stimuli, as LPS (Nayak et al., 2010) and Aβ (Maezawa et al., 2011), which by causing neuronal damage contribute further increase microglia neurotoxicity in AD. In addition to their direct actions via surface receptors, cytokines can induce iNOS expression in microglia, originating high levels of NO that can be neurotoxic (Combs et al., 2001; Heneka et al., 2015; Murphy, 2000). Thus, damage by activated microglia to neurons may occur via NO from iNOS, which inhibits neuronal respiration leading to glutamate release and subsequent excitotoxicity, contributing to neuronal cell death in neurodegenerative diseases (Bal-Price and Brown, 2001), like AD. Also pro-inflammatory cytokine TNF-α can stimulate microglia glutaminase to release glutamate, thus producing excitotoxicity (Takeuchi et al., 2006). Indeed, the glutamate released from microglia when subjected to pro-inflammatory stimuli, is sufficient to promote excitotoxicity in neighbouring neurons. Microglia can produce various defined agonists of excitatory amino acids (EAAs), including glutamate (Barger, 2004), which can mediate microglia activation (McMullan et al., 2012). Under pathological conditions, microglia can upregulate the astroglial glutamate transporter GLT-1 (or EAAT-2), which is known to play a role in glutamate clearance (van Landeghem et al., 2001). However, the presence of ROS or pro-inflammatory cytokines compromises the function of astrocytes in clearing the glutamate released by microglia under physiological conditions (Korn et al., 2005; Trotti et al., 1998). These studies suggest that the relation between neuroinflammation and excitotoxicity in several diseases such as AD has inflammation-excitotoxic intersections (McMullan et al., 2012). Also, Barger and Basile found that microglia activated by APP, but not by Aβ, release high levels of glutamate, which induces excitotoxicity via NMDA receptor signaling, leading to synaptic degeneration and neuronal death (Barger and Basile, 2001).

Extracellular ATP is a prominent signalling molecule involved in several intercellular communications (Ralevic and Burnstock, 1998) that can be released at high concentrations during tissue injury (Zimmermann, 1994). In fact, treatment of microglia with Aβ was shown to cause a fast release of ATP, which was associated to ROS production by microglia (Kim et al., 2012; Kim et al., 2007). The ATP released from damaged neuronal tissues mediate the recruitment of microglia towards the site of injury, in order to minimize the damage. It was suggested that microglia respond to extracellular ATP by further release ATP through lysosomal exocytosis, thus providing a positive feedback reaction to yield a long-distance extracellular signal, leading to migration of distant microglia towards the site of injury (Dou et al., 2012).

In addition, microglia can secret matrix metalloproteinases (MMPs), including MMP9 and MMP2, which are gelatinases implicated in inflammation. These MMPs are responsible for extracellular matrix degradation, impairment of blood-brain barrier (BBB) dynamic properties and inducers of pro-inflammatory cytokines production (Nagase et al., 2006; Nakanishi, 2003), reason why they are considered to also being involved in AD (Wang et al., 2014). In addition, MMP3, MMP9, MMP12, and MMP13, have likewise been demonstrated to be released by Aβ-induced microglia (Ito et al., 2007; Wang et al., 2014). Previous studies have suggested an association between MMP2 activation and ageing (Wang et al., 2003), as well as with myelin degradation (Ihara et al., 2001) and BBB disruption (Liu et al., 2012a). Increased expression of MMP2 in the microvasculature of AD mice models and in brain sections of AD patients was also observed (Grammas et al., 2011). In turn, despite the association of MMP9 with Aβ degradation (Aston-Mourney et al., 2013; Backstrom et al., 1996), MMP2 seems to be also able to degrade Aβ (Merlo and Sortino, 2012; Roher et al., 1994), and their dual importance to amyloid deposition was further corroborated in animal models (Miners et al., 2008; Yin et al., 2006).

In parallel with these mediators related with neuroinflammation, microglia can also release anti-inflammatory cytokines, like IL-4, which can exert neuroprotective role through inhibition of IFN-γ and the consequent decrease in the levels of TNF-α and NO (Chao et al., 1993). In addition, although Aβ do not directly induce IL-10 production by microglia *in vitro* (Franciosi et al., 2005), when cells were pre-treated with IL-10 it showed to inhibit Aβ-induced production of pro-inflammatory cytokines (Szczepanik et al., 2001). Also, the microglial release of TGF-β, a cytokine with pleiotropic functions, revealed to have an anti-apoptotic role and to promote neuronal survival (Meraz-Rios et al., 2013). However, it is believed that the uncoupling of TGF-β signal transduction pathway with inhibition of SMAD3 activation is involved in neurodegenerative diseases (Konig et al., 2005; von Bernhardi et al., 2015).

2.2.3. Microglia migration ability

Microglia display chemokinesis (Vincent et al., 2012), being highly active in their surveilling state, continually surveying the microenvironment with their motile processes and protrusions (Nimmerjahn et al., 2005). They exhibit a dynamic behaviour involving rapid process motility and cellular migration (Damani et al., 2011). In the presence of injury, microglia retract their branching processes and migrate to the site of injury in order to repair CNS homeostasis. Migration, also called chemotaxis, either to the site of neuroinflammation or of Aβ accumulation, is mediated by chemotactic molecules originated in the site of injury, namely ATP, as well as by damaging cells (Gyoneva et

al., 2009; Rubio-Perez and Morillas-Ruiz, 2012). Microglia constitutively express several chemokine receptors (Noda and Suzumura, 2012), including α-amino-3-hydroxyl-5-methyl-4-isoxazoleproprionic acid (AMPA), DAMP, metabotropic P2Y12 purinoceptors and metabotropic glutamate receptors, which mediate their direct chemotaxis (Haynes et al., 2006; Liu et al., 2009; Lynch et al., 2010). Chemotactic response was observed after local injection of ATP in intact brain (Davalos et al., 2005) and in *in vitro* co-culture system, where ATP promoted the migration of microglia across the brain endothelial cell monolayer associated to MMPs secretion that act as a chemoattractant (Maeda et al., 2016). Although ATP is the most used chemoattractant, some other molecules have been proven to control microglia movement, such as adenosine diphosphate (ADP) (Davalos et al., 2005), neuregulin(NRG)1, fractalkine and chemokines, like chemokine motif ligand(CCL)2 (Bose et al., 2016; Calvo and Bennett, 2011). Aβ was similarly indicated to induce a chemotactic response and extensive clustering of microglia around Aβ plaques in the AD brain (Lue et al., 2001). Interestingly, NO seems to be a signal, produced both directly at the lesion and likely by the glial calcium wave, that regulates microglia movement to the site of injury, though without chemoattractant potential (Parkhurst and Gan, 2010; Samuels et al., 2010).

2.2.4. Microglia phagocytic ability

Phagocytosis of pathogens, of cellular debris and of unwanted extracellular protein aggregates is performed by microglia, given that they share some properties of macrophages (Ransohoff and Perry, 2009; Vilhardt, 2005). In the CNS, microglial phagocytic reaction is initiated by the release of attractive signals, designed as “come-get-me” or “find-me” signals (Kettenmann et al., 2013; Neher et al., 2012), for which microglia express a wide variety of receptors (Southam et al., 2016). Such signals lead to the recruitment of microglial cells towards the insult (Echeverria et al., 2016; Nimmerjahn et al., 2005). After recognition of cell-surface signals, or “eat-me” signals, on the target cells, microglia initiate their uptake (Kettenmann et al., 2013; Neher et al., 2012). ATP released from apoptotic or damaged cells acts as a powerful signal for microglial phagocytosis, triggering the purinergic receptor P2Y2 (Elliott et al., 2009). Also, fractalkine released by damaged cells as neurons is recognized by the microglial chemokine (C-X3-C motif) receptor 1 (CX3CR1), promoting microglial phagocytosis (Noda et al., 2011). In addition, phosphatidylserine (Ps) is expressed on plasma membrane of apoptotic cells (Noda and Suzumura, 2012), which may be recognized by Ps receptor (PsR), through MFG-E8 (Hanayama et al., 2002), triggering also phagocytosis (Noda and Suzumura, 2012). Under normal conditions, Ps is located in the inner leaflet of the cell membrane. However, in the course of several inductive factors,

Ps may flip to the extracellular membrane of an apoptotic cell and be recognized by specific receptors on microglia, with a direct binding or by indirect interaction promoting cell phagocytosis (Neher et al., 2012). One example is the connection to vibronectin receptor αvβ3 (VR) through the bridging protein MFG-E8 (Neher et al., 2012). The removal of apoptotic cells and of degenerated neuronal debris in the CNS is the main function of microglia (Colton, 2009) that express MFG-E8 (Fuller and Van Eldik, 2008; Noda and Suzumura, 2012). Phagocytosis is crucial to reduce inflammation and maintain neuronal homeostasis (Noda and Suzumura, 2012). Microglia also express a variety of scavenger receptors (Wilkinson and El Khoury, 2012), and receptors for several components of the complement system (Kettenmann et al., 2011).

According to the type of receptor that is stimulated, phagocytic microglia can produce pro-inflammatory or anti-inflammatory mediators (Napoli and Neumann, 2009), thus presenting a neurotoxic or a neuroprotective role. During uptake of pathogens via TLRs, phagocytosis is associated with inflammation but phagocytosis of apoptotic cells occurs without associated inflammation via PsRs (Neumann et al., 2009). Many of the surface receptors expressed by microglia regulate more than one microglial response beyond the phagocytosis, as follows: CCR1 is the migration-inducing receptor also associated with phagocytic activity; CCR2 and CCR5 are the migration-inducing receptors that induce an anti-inflammatory response; CX3CR1 collaborates in migration, phagocytosis and anti-inflammatory response; triggering receptor expressed on myeloid cells(TREM)2 and MFG-E8 promote phagocytosis and anti-inflammatory response; CD47 and CD200R normally induce phagocytosis under pathological conditions, leading indirectly to an anti-inflammatory response; TLR9 induces phagocytosis and production of pro- and anti-inflammatory substances; and some receptors, such as CD14, CD36, RAGE, TLR1, TLR2, TLR4, and TLR6, induce not only phagocytosis but also inflammatory response (Noda and Suzumura, 2012).

In what concerns AD, it has been demonstrated the ability of microglia to phagocytose and degrade Aβ (Lee and Landreth, 2010; Morgan, 2009), preventing the senile plaque expansion (Bard et al., 2000; Sha et al., 2014). However, an insufficient clearance by microglia have also been suggested to be prevalent in this neurodegenerative disease, leading to increased Aβ accumulation (Cherry et al., 2014; Napoli and Neumann, 2009), observed both in AD patients (Streit, 2004) and in AD transgenic mice (El Khoury et al., 2007). In some studies with microglia in *in vitro* cell cultures it was demonstrated that the cell was able to phagocytose Aβ, though ultrastructural analysis of tissues from AD patients was unable to evidence amyloid fibrils in the lysosomal compartments of local microglia (Meraz-Rios et al., 2013). These

findings suggest that microglia phagocytic capacity may depend on the Aβ species, once other *in vitro* studies reinforced an effectiveness of microglia in degrading the Aβ that was phagocytosed (Lee and Landreth, 2010). Otherwise, it was observed an unusual presence of macrophages infiltrating from the periphery with Aβ within lysosomal compartments (Meraz-Rios et al., 2013). These macrophages that are able to cross the BBB are recruited into the CNS by chemokines and specific cytokines released by microglial and astrocytic activated cells (Meraz-Rios et al., 2013). It has been suggested that such macrophages may be more efficient in Aβ removal and degradation than brain microglia, probably due to the higher acidity of their lysosomes comparatively to those of microglia (Majumdar et al., 2008; Majumdar et al., 2007). However, this macrophage infiltration is thought to only occur under specific conditions, as those of neurodegenerative diseases, such as AD (Ginhoux et al., 2013; Lee and Landreth, 2010). Even so, this issue is still controversial, due to the difficulty in distinguishing macrophages from the activated microglia. Actually, specific immunophenotypic markers for each type of cell are still missing (Lee and Landreth, 2010).

In sporadic cases of AD, insufficient removal of Aβ has been reported as a main pathogenic pathway (Mawuenyega et al., 2010). Pathologic conditions may promote chronic activation of phagocytic pathways that can overburden microglia, thus triggering impairment of their phagocytic activity and leading to a deficient clearance of Aβ in the AD brain (Hickman et al., 2008). It was shown that the activation of TLR2 on microglia increases Aβ phagocytosis (Chen et al., 2006). Other studies demonstrated that microglia require CD14 for phagocytic uptake of Aβ (Reed-Geaghan et al., 2009). Nevertheless, both TLR2 and TLR4 should contribute for Aβ-stimulated phagocytosis (Cameron and Landreth, 2010; Reed-Geaghan et al., 2009), because TLR4 mutation was shown to exacerbate the Aβ load in AD mouse models (Tahara et al., 2006). Besides the direct influence of Aβ in microglia phagocytic property, increased pro-inflammatory cytokines also downregulate the expression of the receptors involved in Aβ phagocytosis, contributing to inefficient microglial phagocytic ability (Hickman et al., 2008; Koenigsknecht-Talboo and Landreth, 2005). Among surface receptors expressed by microglial cells, CD14-TLR2-TLR4 and CD36-TLR2-TLR6 synergistic signalling pathways are fundamental to Aβ-promoted inflammatory response (Noda and Suzumura, 2012). Indeed, phagocytosis may occur together with neuroinflammation in chronic neurodegenerative diseases as AD (Fuller and Van Eldik, 2008; Hoarau et al., 2011; McArthur et al., 2010). In this case increased neurotoxicity may arise due to the production of pro-inflammatory cytokines, NO and ROS (Mizuno, 2012).

As mentioned before, Aβ phagocytosis by microglia depends on its physical and biochemical characteristics. Pan and colleagues have shown that Aβo triggers a potent inflammatory response, with alteration of microglial phagocytic function and deficient removal of Aβf, thus contributing to neurodegeneration in AD (Pan et al., 2011). In fact, while Aβf increased microglial phagocytosis in a dose- and time-dependent manner, Aβo decreased Aβf-stimulated phagocytosis of microglia, suggesting that microglia have a distinct phagocytic response to these two different Aβ forms (Pan et al., 2011). Thus, while microglia internalize Aβo through a nonsaturable, fluid phase macropinocytic process that is distinct from receptor-mediated endocytosis (Mandrekar et al., 2009), microglia respond to Aβf through an Aβ cell surface receptor complex that includes the scavenger receptor CD36, α6β1 integrin, and CD47 (Koenigsknecht and Landreth, 2004), allowing Aβf engulfment and degradation by the endolysosomal pathway (Heneka et al., 2015). Contrasting with Aβf that is usually resistant to enzymatic degradation, Aβo was shown to be degraded by extracellular proteases (Lee and Landreth, 2010), like neprilysin (NEP) and insulin-degrading enzyme (IDE) (Heneka et al., 2015).

The ability of microglia to phagocytose may also depend on the phenotype acquired by the cell, in that the surveilling microglia is supposed to not be involved in phagocytic activity (Streit et al., 1999), while M1 microglia is indicated to be less able to efficiently uptake and degrade Aβ than the M2 microglia (Cherry et al., 2014). However, the M1 classical activation induced by LPS was suggested to trigger Aβ clearance (Herber et al., 2007). Therefore, further studies should explore the differentiated expression patterns of the phagocytic microglia subtype(s).

MFG-E8, whose expression is reduced in AD is also suggested to be involved in Aβ phagocytosis (Noda and Suzumura, 2012). Also, Beclin 1 protein is impaired in AD, and the decrease of its expression *in vitro* and *in vivo* was shown to interfere with the phagocytic efficiency, leading to decreased receptor recycling of CD36 and TREM2 (Lucin et al., 2013), which contribute to reduced Aβ uptake (Southam et al., 2016).

Another important microglial functional property in sustaining brain homeostasis is the synaptic pruning (Chen et al., 2014), which contributes to control synapse density (Ji et al., 2013) and regulate network activity (Southam et al., 2016). Microglia prune synapses as an extension of normal phagocytosis (Southam et al., 2016), what depends on neuronal activity (Schafer et al., 2012). Curiously, Aβ is suggested to mediate increased synaptic pruning (Kim et al., 2013).

Finally, it is important to note that under certain conditions, such as inflammation, microglia can also phagocytose viable neurons, leading to neuronal death. In fact, although high concentration of Aβ (μM) can induce neurotoxicity, in mixed neuronal-glial

cultures low Aβ concentrations (nM) promote neuronal loss through a microglia-mediated mechanism, suggesting that microglial phagocytosis is the primary cause of neuronal death induced by nanomolar concentrations of Aβ (Neniskyte et al., 2011). Externalization of Ps by neurons was shown to be induced by Aβ exposure (Mohmmad Abdul and Butterfield, 2005) and the number of Ps-exposing neurons was found increased in both AD and MCI (Bader Lange et al., 2008), thus inducing their phagocytosis.

2.3. Microglia activation *vs* dysfunction during AD progression and ageing

Nowadays, the effective role of microglia in AD progression remains unsolved. However, it is known that microglial activation, depending on the stimulus, can promote neuroprotective or neurotoxic responses contributing to slow or accelerate disease progression (Hanisch and Kettenmann, 2007; Schwartz et al., 2006). As previously mentioned, microglia may be activated by Aβ (Mizuno, 2012). The presence of activated microglia near amyloid plaques suggests that Aβ accumulation may trigger a persistent microglial activation (Heneka et al., 2013). Consequently, microglia may become over-activated and produce pro-inflammatory factors that are prejudicial to neuronal cells, triggering the release of alarmin molecules by damaged neurons which in turn will activate more glial cells creating a vicious cycle that intensify the generation of more inflammatory signals (Glass et al., 2010; Mizuno, 2012). This process of inflammation may contribute to AD progression by increasing neuronal death (Solito and Sastre, 2012). However, it is possible that early in AD disease microglia may have a more protective instead of a detrimental role, with a prevalent phagocytic phenotype that allows a more efficient removal of Aβ by producing increased amounts of enzymes involved in Aβ degradation (Agostinho et al., 2010; Hickman et al., 2008). It was also suggested that microglia may become dysfunctional instead of a gain in inflammatory function, losing their capacity to support neurons and to remove neuronal debris or Aβ, favoring neurodegeneration (Streit and Xue, 2012). All these alterations on microglial function contributing to disease progression may be due to ageing (Streit, 2006).

In studies carried in the last decade, Damani and colleagues reported that the ageing phenotype of microglia involves alterations in their morphology and dynamic behavior (Damani et al., 2011). They found that aged microglia in resting/surveilling state have significantly smaller and less branched processes, and slower process motilities, which probably compromise their ability to survey and interact with their environment continuously. They also found that dynamic microglial responses to injury were age-dependent. While young microglia responded to extracellular ATP by increasing their motility and become more ramified, aged microglia exhibited a contrary response

becoming less dynamic and ramified. Microglia proliferation is also suggested to increase with ageing (Lasiene et al., 2009), but microglia activation and repeated rounds of replication may also promote replicative senescence (Conde and Streit, 2006; Streit, 2006). Indeed, Streit and colleagues have found a morphological aged phenotype that differs from microglia activation, which they designated as dystrophic or senescent microglia (Streit et al., 2004). This dystrophic phenotype is characterized by cytoplasmic structure abnormalities with deramified, atrophic, fragmented, and abnormally-twisted processes (Streit, 2006; Streit et al., 2004). It is important to note that fragmentation of the microglial cytoplasm (cytorrhexis) is not accompanied by nuclear fragmentation (karyorrhexis), which is typical of apoptosis (Streit, 2006). Also, work in the aged human brain has provided evidence of structural deterioration of microglia (Streit, 2002), suggesting its association with age-related brain dysfunction (Olah et al., 2011; Streit et al., 2004). Another sign of senescence is the shortening of telomere that was observed both in *in vitro* and *in vivo* microglial cells overtime (Flanary and Streit, 2003; Flanary and Streit, 2004). Accordingly, chronic activation of microglia during long periods could lead to their over-activation followed by degeneration of microglial cells (Graeber and Streit, 2010), which can occur sporadically throughout the aged human brain (Streit and Xue, 2009). These observations raise the hypothesis that ageing adversely affect viability and self-renewal capacity of microglia, resulting in the generation of senescent and/or dysfunctional cells (Streit, 2006), that compromise the neuroprotective role performed by microglial cells (Olah et al., 2011).

Overall, these altered features of microglia behavior at rest and following injury reveal an age-dependent dysregulation of immune response in the CNS that can facilitate the emergence of AD. Indeed, these aged microglia morphological abnormalities seem to be more pronounced in AD (Streit, 2002). It is well established that AD is an age-related neurodegenerative disease (Lynch et al., 2010). In fact, ageing shares many pathophysiological conditions with AD, such as increased oxidative stress and inflammation, with increased levels of cytokines and glial cell reactivity (Akiyama et al., 2000; Demarin et al., 2009; Fratiglioni and Qiu, 2009; Tan et al., 2007). Additionally, Aβ-removal mechanisms decrease with age (Akiyama et al., 2001), and during AD (Caccamo et al., 2005). Thus, it is believed that changes on microglial phenotype and function during ageing have an important role in neurodegenerative diseases (Streit and Xue, 2009; von Bernhardi et al., 2011). Indeed, the degeneration of microglial cells is highly increased in AD, where severely dystrophic microglia, rather than activated microglia, are colocalized with degenerating neuronal structures, further supporting the idea that neurodegeneration could be a consequence of reduction of microglial neuroprotection (Streit et al., 2009; Streit and Xue, 2009). However, neuronal loss and

cognitive decline are features of an aged healthy brain that have been also related with microglial activation. Post-mortem studies that indicate the presence of activated microglia in aged brains may derive from some degree of neuronal damage during normal ageing (Schuitemaker et al., 2012). Thus, the progressive ageing-related microglial degeneration and loss of microglia neuroprotection together with chronic microglial activation may contribute to the onset of neurodegeneration (Streit et al., 2009). The accumulation of dystrophic microglia may derive from increased cell vulnerability to several factors that may include the presence of Aβ, although its prevalence was shown to increase by amyloid load and to favor AD neurodegeneration (Flanary et al., 2007; Graeber, 2010; Miller and Streit, 2007; Streit, 2006).

Although phagocytic activity of microglia obtained from young and aged rats seems to increase with age (Lynch et al., 2010), the decreased microglial phagocytic capacity towards Aβ and the lower microglia migratory capacity (Njie et al., 2012; Pan et al., 2011), may result in amyloid accumulation (Floden and Combs, 2011; Lynch et al., 2010). Further studies indicate microglia cannot efficiently degrade Aβ aggregates in aged brains, what favors senile plaque accumulation mainly in the elderly (Echeverria et al., 2016). These issues may relate with a downregulation of receptors related with Aβ phagocytosis, such as CD36, and RAGE, and a decrease of Aβ degradation enzymes, namely MMP9, observed in old non-disease mice (Hickman et al., 2008).

Furthermore, aged microglia, although producing elevated pro-inflammatory cytokines, namely IL-6 and TNF-α, when compared to younger cells, are less responsive to stimulation (Njie et al., 2012). Progressive age-related MHC class II and CD11b expression support the idea that microglial activation occurs during ageing (Streit et al., 2004; Wong, 2013), together with upregulated mediators indicative of both inflammation and oxidative stress (Lynch et al., 2010). Interestingly, it was also suggested that microglia lose their lysosomal capacity due to changes in cathepsin activity, promoting a decrease in autophagic and mitochondrial activity, which subsequently leads to less ATP and more ROS production, culminating in an energy deficit and oxidative stress (Ralay Ranaivo et al., 2006). Dystrophic microglia may additionally contribute to neurodegeneration due to their reduced phagocytic function and autophagy (Olah et al., 2011). This situation may induce neighboring microglia activation, with increased release of inflammatory factors and more neuronal cell death, thus originating a vicious cycle that can aggravate disease progression (Olah et al., 2011).

Interestingly, microglial cells were described to be overactivated increasingly reacting to inflammatory stimuli in the ageing CNS of mice, rats, and primates, similar to that of microglia in brains with progressive neurodegeneration (Perry and Teeling, 2013). This phenomenon, known as priming, is characterized by a more immediate and

exacerbated production of inflammatory cytokines and ROS (Heneka et al., 2015). This state of activation seems to be related with increased expression of TLRs and MCH class II (Olah et al., 2011). All of these alterations in the dynamic microglial behavior with ageing may therefore contribute to AD progression in the ageing CNS.

Another evidence of microglia neuroprotective loss derives from their switch from M2 to M1 phenotype during ageing in the APP/PS1 AD mouse model (Jimenez et al., 2008), with less responsiveness to M2 induction factors (e.g. IL-4) suggested to be related with an age-associated decrease in IL-4Rα (Fenn et al., 2012). These findings that include age-related alterations on microglia Aβ-induced inflammation, as well as decreased microglia ability to switch phenotypes and attenuate injury (Cherry et al., 2014) may create the conditions fro AD onset and progression.

2.4. Microglia-neuron interplay in AD

Microglia have the function to protect and support neuronal cells but their immune function are regulated by inhibitory factors intrinsic to the CNS (Streit, 2002). In fact, neurons release several signals, named “on” and “off” signals that can modulate microglial activation (Biber et al., 2007), involving multiple mechanisms with different type of action (Olah et al., 2011). Thus, an imbalance of this regulation may contribute for the age-related alterations and vulnerabilities observed in neurodegenerative processes (Wong, 2013). Accordingly to this, several studies suggested that to maintain the microglia surveillance phenotype it is required their interaction with neurons. Indeed, it is known that under physiological conditions neurons produce a lot of inhibitory or calming signals to prevent unwanted damage of neurons (Gemma and Bachstetter, 2013), namely chemokine (C-X3-C motif) ligand 1 (CX3CL1 or fractalkine), CD200 and CD47 (**Figure 1.6)**. These factors can modulate the response of microglia and decrease their activation (Clark et al., 2007; Puntener et al., 2012; Streit et al., 1999; Tambuyzer et al., 2009), by their binding to receptors on microglial cells (Kierdorf and Prinz, 2013).

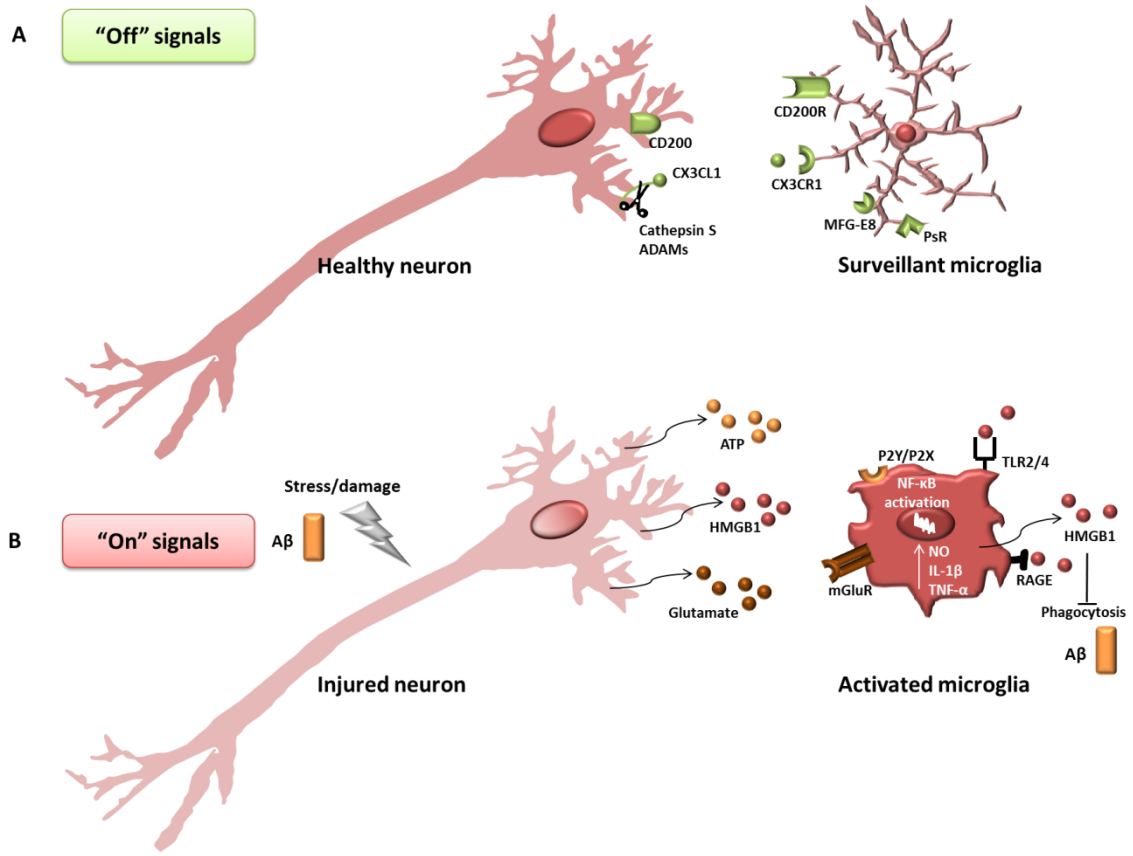

Figure 1. 6 – Microglia-neuron signalling pathways that regulate microglial responses in AD. (A) To maintain microglia in a surveillant/quiescent state, neurons inhibit microglia activation through the cell-cell contact (CD200-CD200R) and the release of "off" signals, such as chemokine (C-X3-C motif) ligand 1 (CX3CL1 or fractalkine) mediated by metalloproteinases (ADAMs) or cathepsin S, through its exclusive receptor CX3CR1 on microglia. CX3CL1 released by neurons increases the removal of neuronal debris by microglia, mediated by phosphatidylserine receptor (PsR) and milk fat globule factor-E8 (MFG-E8) recognition. **(B)** In case of injury, degenerated neurons release mediators that activate microglial cells, like adenosine triphosphate (ATP) which is recognized by P2Y and P2X receptors in microglia, and glutamate, which is recognized by both ionotropic and metabotropic glutamate receptors (mGluRs) in microglia, inducing a neurotoxic or neuroprotective response depending on the receptor stimulated. Also the alarmin high-mobility group box 1 (HMGB1) released by activated microglia and injured neurons contribute for a vicious cycle regulating chronic, progressive degeneration related with neuroinflammation. HMGB1 may activate microglia through the receptor for advanced glycation end products (RAGE) and toll-like receptors (TLRs) 2 and 4, triggering downstream signalling cascades, like nuclear factor-κB (NF-κB) activation that induces the transcription of inflammatory cytokines. HMGB1 can also inhibit amyloid-β (Aβ) peptide phagocytosis by microglia, further contributing to AD progression. Moreover, Aβ may lead to the release of several inflammatory factors by microglia, like oxide nitric (NO), promoting neurotoxicity.

The chemokine fractalkine is one of the best-characterized examples of a neuronal mediator, and its soluble form mediates microglial chemoattraction, while its membrane-anchored form promotes cell adhesion (Gemma and Bachstetter, 2013; Noda and Suzumura, 2012). Fractalkine is highly expressed by healthy neurons (**Figure 1.6)**, allowing the modulation of microglial function through its exclusive receptor

CX3CR1, which is basically expressed in microglia keeping microglia in a surveillant/ramified state (Gemma and Bachstetter, 2013; Noda and Suzumura, 2012). Indeed, several evidences indicate that fractalkine can repress the activation of microglial cells (Gemma and Bachstetter, 2013), and microglia-induced neurotoxicity (Noda et al., 2011). CX3CL1 directly influences several microglial functions, namely migration, proliferation, suppression of glutamate-induced neurotoxicity and inhibition of pro-inflammatory cytokine production (Noda and Suzumura, 2012). Soluble fractalkine released by neurons directly increases microglial removal of degenerated neuronal debris, which is regulated by PsR and MFG-E8 (Noda et al., 2011). Indeed, MFG-E8 is upregulated in microglial cells after detection of neuronal fractalkine signaling leading to recognition of apoptotic neurons by microglial cells (Leonardi-Essmann et al., 2005). The membrane-anchored CX3CL1 is cleaved by various proteases, such as a disintegrin and ADAM family metalloproteinases (ADAM10 and ADAM17), and cathepsin S (Noda and Suzumura, 2012). Interestingly, it was observed that levels of fractalkine, which are highly expressed in young healthy rat brains, were diminished in aged rats probably favoring neuroinflammation. In fact, fractalkine administered to aged rats reestablished microglial cells in a more ramified morphology (Bachstetter et al., 2011). Selective activation of CX3CR1 was also shown to increase synaptic strength suggesting that neuronal fractalkine may regulate synaptic strength (Clark et al., 2015). In contrast, impairments in synaptic plasticity and cognitive function, originated by an increased microglia activation and inflammation in the CNS, together with increased tau pathology, were suggested to be due to fractalkine/CX3CR1 signaling loss (Bhaskar et al., 2010; Rogers et al., 2011). Other studies evidenced that loss of microglia-neuron fractalkine signaling prevented Aβ accumulation in mouse models of AD, in the course of cell activation and increased phagocytic ability of CX3CR1-deficient microglia (Fuhrmann et al., 2010; Fuhrmann et al., 2011; Lee et al., 2010). Therefore, CX3CR1 is relevant to modulate microglia functions in neurodegenerative diseases (Cardona et al., 2006; Lee et al., 2010). Even so, the importance of CX3CL1-CX3CR1 in AD is still controversial and needs further clarification.

The CD200-CD200R interaction (**Figure 1.6)** has similarly been related with microglial-neuronal communication (Hanisch and Kettenmann, 2007). CD200 is a transmembrane glycoprotein expressed by neurons that exerts inhibitory regulation of CD200R in microglia (Greter and Merad, 2013; Koning et al., 2009; Noda and Suzumura, 2012). Both molecules are expressed and distributed throughout the brain (Varnum and Ikezu, 2012). CD200 was shown to have a pivotal role in both autoimmune response and inflammation in AD (Varnum and Ikezu, 2012). CD200-CD200R interaction leads to an anti-inflammatory state that protects against neurotoxic stimuli (Noda and Suzumura,

2012). Addition of neuronal cells to LPS-treated or Aβ-treated microglial cultures was demonstrated to suppress the expression of surface markers of microglia and secretion of inflammatory cytokines, a condition that was inhibited by the anti-CD200 antibody (Lyons et al., 2007a; Lyons et al., 2009). Intriguingly, it was observed an inverse correlation between CD200 expression and the expression of cell surface receptors of microglial activation by age (Lyons et al., 2009), either in Aβ-treated rats (Lyons et al., 2009), or in AD patients (Lynch et al., 2010), suggesting that suppression of CD200-CD200R axis is involved in AD pathology (Walker et al., 2009). CD200 deficient mice show a profound impairment in synaptic plasticity in hippocampal slices and increased microglial activation (Costello et al., 2011). In aged AD animal models with increased microglial activation and Aβ-induced reduced in CD200 expression, IL-4 was shown to restores such deficit (Kiyota et al., 2010; Lyons et al., 2007a; Lyons et al., 2007b). Actually, IL-4 is deficient in AD models favoring increased inflammatory responses to LPS (Lyons et al., 2009). IL-4 revealed to enhance the removal of Aβ and to improve spatial learning in APP/PS1 mice, but only in the pre-symptomatic stages of the disease (Kiyota et al., 2010), suggesting that the decreased CD200 expression in the aged and AD brain may compromise the IL-4 signalling at later stages of disease (Varnum and Ikezu, 2012).

CCL21 is another neuronal chemokine that is upregulated in degenerating neurons and triggers microglia recruitment through CXCR3, which also interacts with the neuronal CXCL10 regulating microglia migration (Noda and Suzumura, 2012). Thus, CCL21 and CXCL10 synergistically promote microglia migration through the receptor CXCR3, having CXCL10 the ability to suppress CCL21-induced migration in microglia through such receptor (Noda and Suzumura, 2012).

Moreover, microglia may be activated by molecules derived from degenerated neurons, namely when exposed to Aβ aggregates (Mizuno, 2012; Streit et al., 1999; Varnum and Ikezu, 2012). Injured neurons release several signalling factors (**Figure 1.6)**, known as find-me, eat-me, and help-me signals (Noda and Suzumura, 2012), or just “on” signals (Biber et al., 2007), such as nucleotides, cytokines, and chemokines, to recruit and activate microglial cells (Biber et al., 2007; Koizumi et al., 2007). This microglial activation may produce trophic or other factors that promote neuronal recovery (Streit, 2005), but may also contribute to exacerbate neuronal death due to release of neurotoxic mediators by microglia, further suggesting that microglial cells are responsible for the highly neurodegeneration that occurs in AD (Ralay Ranaivo et al., 2006). However, during neurodegeneration, the injured neurons may remain viable and trigger microglial activation. Reversible neuronal injury induce the release of trophic factors by

microglial cells as survival-promoting signals (Streit et al., 1999), with protective and remodeling role (Parkhurst et al., 2013). Irreversible neuronal injury may, otherwise, trigger the production of neurotoxic factors by microglia, as death-promoting signals that contribute to neurodegeneration and subsequent phagocytosis by microglial cells (Streit et al., 1999). Among the signals released by injured neurons (**Figure 1.6)** are purines and neurotransmitters (Biber et al., 2007). Purines like ATP are recognized by the nucleotide receptors P2Y and P2X in microglia (Inoue, 2006). Several works suggest that purines play a crucial role in microglial activation and induce their migration to the site of damage (Koizumi et al., 2013; Sperlagh and Illes, 2007). In addition, it was shown that microglia migration is mediated by the extracellular ATP (Dou et al., 2012). Also the neurotransmitter glutamate is indicated to have an important role in neuron-microglia interplay. Indeed, although glutamate directly may induce neurotoxicity, it also can activate microglial cells, which express both ionotropic and metabotropic glutamate receptors (Kettenmann et al., 2011). So, depending on the stimulated microglia receptor for glutamate it may originate neurotoxicity or neuroprotection (Biber et al., 2007).

Exposure of microglia to Aβ aggregates may also promote the release of several neurotoxic factors (**Figure 1.6)**, such as NO (Maezawa et al., 2011), suggesting a significant contribution of Aβ for microglial-induced neurotoxicity, rather than a direct Aβ-induced neurotoxicity in AD. Also, the microglial Aβ phagocytosis dysfunction caused by HMGB1, a chromosomal protein that inhibits phagocytosis and accumulates extracellularly on Aβ plaques, may be involved in the progression of AD (Takata et al., 2003a; Takata et al., 2012). The “on” signal HMGB1 **(Figure 1.6B)** is an inflammatory factor that acts as an alarmin being released by necrotic or damaged astrocytes, microglia and neurons (Fang et al., 2012; Muller et al., 2001), and during acute inflammatory responses (Park et al., 2004). It seems that HMGB1 has two different functions in cellular systems (Park et al., 2004). Intracellular or nuclear HMGB1 stabilize DNA structure and regulates transcription of several genes, including pro-inflammatory ones (Bianchi and Manfredi, 2009; Park et al., 2009; Wong, 2013). In opposite, during activation or cell necrosis, HMGB1 increase in the cytoplasm and is released to the extracellular space, where it promotes inflammation that contributes to neurodegeneration (Kawabata et al., 2010). These inflammatory responses may be induced through the microglial receptors RAGE, and TLRs 2 and 4 (Park et al., 2006). HMGB1 was suggested to decrease in neurons, while increase in astrocytes with ageing (Enokido et al., 2008).

3. Models to assess microglia activation/Dysfunction profiling

Several models can be used to assess the importance of microglia in the pathogenesis of AD. Indeed, *in vitro* models using pure cultures of microglia allow the exploration of microglia specific molecular mechanisms, while *ex vivo* models, such as the organotypic hippocampal slice cultures, permit the understanding of microglia reactivity within the tissue environment. Moreover, the use of an *in vivo* model of AD is the only suitable way to identify the role of microglia on the initiation and progression of AD. However, the new spheroids with human cells derived from AD-induced pluripotent stem cells (iPSCs) have recently shown to better recapitulate the AD disease.

3.1. *In vitro* and *ex vivo* models

The cell culture systems are important tools to evaluate microglia functionality, including the activation state, released factors and mobility, among others, which cannot be properly evaluated *in vivo* (Stansley et al., 2012). Microglia primary cultures and cell lines of microglia, like murine BV2 and N9, are the cell culture models most frequently used in the laboratory. Indeed, due to their resemblance with *in vivo* cells, the primary cultures are commonly used in studies to evaluate microglial function in AD models, especially on Aβ-related effects (Maezawa et al., 2011; Takata et al., 2003b; Takata et al., 2012). Usually, microglia are isolated from the cortex of rodents, such as mouse or rat, either prior to, or shortly after birth (Stansley et al., 2012). However, because it is a time consuming technique with a relative low cell yield, many studies use microglia cell lines rather than primary cells to assess the microglial role in physiological and pathological conditions (Stansley et al., 2012). The use of cell lines is less expensive and have less variation between different cultures (Ni and Aschner, 2010). Nonetheless, microglia cell lines are genetically modified, like N9 that is derived from mouse and immortalized by oncogene-carrying retrovirus (Righi et al., 1989). Such genetic modifications cause changes in adhesion and proliferative ability of the cells, what makes their inflammatory responses different from primary microglia (Horvath et al., 2008). Nevertheless, they constitute an advantageous tool to explore the role of microglia in inflammation-associated neurodegenerative diseases as AD.

Although cell cultures from young, as well as old human or animal donors, have been considered the most appropriated model (Schneider and Mitsui, 1976), we have already shown that both neurons and astrocytes show maturation and ageing along time in culture (Falcão et al., 2006). These findings indicate that microglia can probably be similarly aged in culture, what may be important to evaluate microglia-induced neurodegeneration. Successive shocks with LPS have also shown to cause microglia ageing (Yu et al., 2012). Until now there is no convenient protocol to obtain reproducible

aged microglia, even from murine models at different ages (Njie et al., 2012). The most senescent do not survive and the isolation procedure may induce activation (Cristovão et al., 2010). Therefore, these aspects should be considered when microglia response to a stress-stimulus is to be determined.

Recently, studies in human stem cell and three-dimensional (3D) culture technologies allowed the development of new 3D neuronal cell culture models that mimic AD pathologies (Choi et al., 2016). These models use AD patient-derived induced pluripotent stem cells (iPSCs) or genetically modified human stem cell lines, which are cultured on a Matrigel that functions as a 3D support matrix providing a brain tissue-like environment (Choi et al., 2014; Choi et al., 2016). In addition, cerebral organoid cultures have also been used to obtain 3D structures to study AD pathology (Choi et al., 2016).

Many studies have been performed in an attempt to better understand the relationship between neurons and microglial cells. Although, a pure microglia culture is the only model to assess cell reactivity when microglia-neuron interplay is intended in what concerns the effects produced by soluble factors released by neurons on microglia reactivity, it may be used the conditioned media model. This microglial and neuronal conditioned media isolated after a given stimulus may be incubated with neurons or microglia to evaluate the functional impairments. Another useful tool to evaluate the interaction between microglia and other cell types is the mixed co-culture system (Stansley et al., 2012) that is important to unravel the role of neuroinflammation in neurodegenerative diseases, such as AD. Indeed, several studies using different co-culture approaches have been performed and reported (Culbert et al., 2006; Gresa-Arribas et al., 2012). They may mimic the *in vivo* environment (Carreira et al., 2012), and the cells may either be in contact, or separated if a transwell co-culture system is used instead. In spite of some drawbacks, the co-cultures system constitute a good tool depending on the study purpose (Carreira et al., 2012).

To mimic an AD condition, several stimuli can be used in *in vitro* models. While some authors have been treating nerve cells with Aβ fragments, namely the $A\beta_{1-42}$, others use Aβo to better relate with the Aβ aggregation that occurs along disease progression (Maezawa et al., 2008; Maezawa et al., 2011). Aβo are small diffusible aggregates, formed by $A\beta_{1-42}$ self-association, that are considered more deleterious than Aβf (Lambert et al., 1998). In addition, also the concentrations of Aβo used may differently affect cell response. Aβo at low nanomolar concentrations (5-50 nM) do not elicit neurotoxicity but promote a significant increase in cell immunoreactivity for SRA and CD11b, markers of activated microglia (Bornemann et al., 2001). However, Aβo

concentrations ranging from 100 nM to 10 µM were found to cause neuronal death (Nicholson and Ferreira, 2009) and reduced survival of mature oligodendrocytes (Horiuchi et al., 2010). Interestingly, recent studies show that nanomolar concentrations of Aβ promote microglia phagocytosis of viable neurons and synapses in culture, which may modulate neuronal loss and the progression of AD (Neher et al., 2012; Neniskyte et al., 2011). Taking this in consideration, it is likely that Aβo can reach the low nanomolar concentrations required for microglia activation in the first steps of AD development and progressively accumulate to higher and more neurotoxic levels with disease progression giving rise to the specific AD symptoms (Hardy and Selkoe, 2002).

Although microglia cross-talk with other nerve cells may be studied using the conditioned media or co-culture model, the interplay between the different nerve cells, the matrix and tissue organization may only be studied using a more complex model, the organotypic slice cultures. These cultures are reported as the most approximate model to the *in vivo* conditions since it allows interaction between neighbouring cells, and also the maintenance of three dimensional architecture of the brain (Humpel, 2015), which is not achieved in primary cultures. In addition, organotypic hippocampal slice cultures have been considered an alternative to animal model experiments. Cultured slices maintain the cell architecture. They have been extensively used to investigate molecular mechanisms involved in cytotoxicity, such as the ones that are determined by Aβ toxicity (Kreutz et al., 2011), as well as to evaluate neuroprotective strategies. Although the organotypic slice cultures are usually prepared from young animals (5-12 day-old pups) to better preserve the tissue characteristics and functionality (Su et al., 2011), adult animals are the most appropriate to mimic AD disease (Brewer, 1997), and studies already used organotypic cultures from two months old rodents (Staal et al., 2011; Wilhelmi et al., 2002), or even from 14-16 months old rodents (Schrag et al., 2008).

3.2. *In vivo* models

In vivo models are the most suitable to study disease phenotype and progression. Several mouse models have been generated in an effort to express similar human AD behavior and neuropathology as well as to validate new therapeutic approaches. Such transgenic animals that mimic the etiology and the progression of the disease, should not have symptoms that may alter the disease diagnosis (Do Carmo and Cuello, 2013).

Initially, transgenic mice models of AD have been created by introducing mutated forms of the human APP and/or presenilins genes found in familial AD cases (Schwab et al., 2009). Even though the early-onset of FAD only represents less than 5% of total AD cases, there is a broad phenotypic similarity between FAD and LOAD, suggesting

that findings about FAD would also be directly relevant for LOAD (Selkoe, 2001). Interestingly, while the introduction of wild-type forms of APP and PS1, as found in normal and non-familial AD cases, does not cause pathology, the transgenic mice with pathological human mutant proteins easily show Aβ aggregation, behavioral deficits and some abnormal tau-phosphorylation (Duyckaerts et al., 2008; Howlett and Richardson, 2009; Morrissette et al., 2009). However, these models do not exhibit the development of neurofibrillary tangles or massive neuronal loss, which are the major pathological hallmarks of AD (Schwab et al., 2009). Thus, additional pathological tau mutations have been created to mimic this AD feature (Oddo et al., 2003b).

3.2.1. A triple transgenic mouse model of AD (3xTg-AD)

Among the transgenic mouse models created, just a small number express both plaques and tangles pathologies that characterize AD (Sterniczuk et al., 2010b). The 3xTg-AD mice mimic the behavior and the neuropathological features of human AD patients. The 3xTg-AD mice express three mutations in a comparable manner to that observed in AD patients (Mesulam, 2000): human amyloid precursor protein Swedish (APP_{Swe}), human presenilin-1 M146V ($PS1_{M146V}$), and the P301L mutation of human tau (tau_{P301L}), allowing the development of amyloid plaques, acceleration of the deposition rate, and neurofibrillary tangle pathology, respectively, in a temporal and spatial progression that mimics the pathogenic stages observed in humans (Oddo et al., 2003a; Oddo et al., 2003b; Sterniczuk et al., 2010a; Sterniczuk et al., 2010b). First hallmarks arise in hippocampal, amygdala and cortical regions, which are the most affected regions by AD pathology. In fact, in 6 months 3xTg-AD mice deposition of extracellular Aβ is apparent in frontal cortex (Oddo et al., 2003a), that continues to spread into the hippocampus by 12 months (Gimenez-Llort et al., 2007). Differently, tau immunoreactivity is only firstly observed at 12 months in pyramidal neurons in the CA1 region (Oddo et al., 2003b). Thus, 3xTg-AD animals develop extracellular Aβ aggregates prior to tangle formation, corroborating the amyloid cascade hypothesis considering Aβ as the trigger of the disease (Oddo et al., 2003a; Oddo et al., 2003b).

At 2-months of age no Aβ or tau pathology is observed in any brain region of the 3xTg-AD mice (Oddo et al., 2003b), as well as no impairment in learning or memory (Billings et al., 2005). The earliest cognitive impairment manifests at 4-months with retention for spatial and contextual learning. This is in agreement with the presence of intraneuronal Aβ in the hippocampus, cortex and amygdala at that age, suggesting its role as promotor of cognitive decline onset in the 3xTg-AD mice (Billings et al., 2005). Extracellular Aβ deposits are not visible at this age. At 6-months 3xTg-AD animals show no apparent tangle pathology, but contain diffuse Aβ plaques in cerebral cortex, and

intraneuronal Aβ deposits within pyramidal neurons of the hippocampus, cortex and amygdala (Billings et al., 2005; Oddo et al., 2003b), followed by microgliosis and astrogliosis (Caruso et al., 2013; Janelsins et al., 2008). At 9-months the hippocampus of the animals is virtually plaque-free, becoming remarkable at 12-months (Oddo et al., 2003a; Rodriguez et al., 2010), where apparent conformational alteration in tau spreads by the cerebral cortex (Oddo et al., 2003a; Oddo et al., 2003b). So, the 3xTg-AD model of AD provides a promising tool for understanding the neuropathology of AD, with expression of AD pathology in an age-dependent manner beyond the associated behavioral changes (Sterniczuk et al., 2010b) that occurs with disease progression in human patients (Mesulam, 2000; Vandenberghe and Tournoy, 2005; Witting et al., 1990). Although several studies can be performed with this animal model, organotypic cultures from 3xTg-AD exposed to different stimuli and specific inhibitors are promising to obtain pre-clinical data in a more rapid and less expensive way.

3.2.2. Other in vivo models

Besides the 3xTg-AD model, there is a wide variety of other animal models of AD that provide different approaches depending on the mechanisms intended to be studied. Among them, a widely-used model is the Tg2576 mouse that expresses the human APP695 isoform with Swedish double mutation, showing 6-times more APP then normal mice (Westerman et al., 2002). This model exhibits age-dependent Aβ deposition (Elder et al., 2010), with increased levels at 9-month old (Westerman et al., 2002). In turn, APP mouse model expresses both APP695 (APP_{Swe}) and APP751 isoforms, showing 7-times more APP then normal mice and Aβ plaques at 6-months of age. These two models have been considered important tools to assess the correlation between amyloid deposition and cognitive alterations in AD (Foley et al., 2015). Also PS1 mice model which express human presenilin 1 with M146L or M146V mutation, show high levels of endogenous mouse $Aβ_{42}$ (Weggen and Beher, 2012). Thus, the co-expression of the mutant PS1 gene increases the pathology to a significantly younger age, as evidenced by the double transgenic APP/PS1 model (Borchelt et al., 1997; Holcomb et al., 1998). Another mice model is the APP_{Swe}/PS1deltaE9 that express APP with the Swedish mutation and mutant human PS1 with a deletion of exon 9 (Jankowsky et al., 2001; Savonenko et al., 2005). Interestingly, the amyloid plaques precede typical cognitive impairments in this model (Jankowsky et al., 2004; Volianskis et al., 2010), what allows the evaluation of pathophysiological events associated to preclinical AD (Zou et al., 2016). Another useful model of intraneuronal $Aβ_{42}$ and neurodegeneration is the 5XFAD (Eimer and Vassar, 2013). This mouse model expresses five FAD mutations, two PS1 and three APP mutations, leading to $Aβ_{1-42}$ overproduction and acceleration of plaque development

(Oakley et al., 2006). Consequently, 5XFAD mice develop intraneuronal $A\beta_{1-42}$ at 1.5 months of age, amyloid plaques at 2 months, and significant neuron loss at 9 months, with initial cognitive impairments from 4 months forward (Oakley et al., 2006). However, NFTs are not typical in this model, and neuronal loss only occurs by combination of multiple mutations (Elder et al., 2010).

4. Global aims of the thesis

AD is an age-associated pathology characterized by increased accumulation of extracellular Aβ plaques within the brain. Histological studies have revealed high microglial activation in diseased brains commonly associated with these peptide aggregates. On the other hand, healthy brain ageing is characterized by neuronal loss and decline of cognitive function being inflammation a major causative factor. Inflammation is closely associated with microglial activation, priming and ageing which are now considered to be major drivers for the neuropathological findings in AD. Therefore, this study intends to unveil the role of microglia in AD pathogenesis using primary cultures of young/reactive (acutely isolated microglia) and long cultured (aged) microglia to assess the different representation of microglia subtypes upon Aβ interaction, followed by assessment of microglia-neuron interplay in the 3xTg-AD model.

As a first step, it will be evaluated how the phenotypes of 2 days *in vitro* (DIV) cultured (acutely isolated) and 16 DIV (aged) microglia will mimic the activated and the irresponsive/dormant microglia phenotypes, by evaluating microglia functional properties as autophagy, phagocytosis and migration, morphological features, markers of senescence and expression of inflammatory mediators. This will be an innovative approach to assess how differently activated microglia may diversely respond to stress-stimuli, and whether aged-associated responsiveness may compromise the neuroprotective properties of the cell.

Next, it will be determined how such differently aged and polarized microglia subsets will diversely react to 50 nM and 1000 nM Aβ stress treatment for 24 h in terms of M1/M2 representation and microglia dynamic properties. It will be assessed changes in their morphological characteristics, viability, migration, phagocytic capacity, inflammatory biomarkers, as well as in the release of MMP2, MMP9 and glutamate. With such evaluations it will be possible to obtain the representation of polarized phenotypes in such Aβ-treated cells, in terms of either the classical activated M1 subtype considered as pro-inflammatory and non-phagocytic, or the alternatively activated M2 subtype associated to an anti-inflammatory and phagocytic cell, besides the number of cells that progress to a subclass of senescent-like, which will be related with each one respective specific physiological characteristics.

To complement the *in vitro* studies it will be used the triple transgenic animal model of AD, the 3xTg-AD mice. Two main regions will be evaluated, the hippocampus and the cortex, at 3, 6 and 9 months-old, in order to evaluate markers of early AD disease and characterize the microglia population phenotypes along disease progression, when compared with age-matched wild-type controls.

Altogether, it is expected that the present study will contribute to elucidate the involvement of microglia cells in AD-associated neuroinflammation, and their contribution in the onset and progression of AD, in order to identify possible targets to modulate microglia towards a neuroprotective role, and to establish the most appropriated therapeutic strategies and their implementation timing. Indeed, we hypothesize that distinct therapeutic strategies may be required depending on the stage of disease progression.

5. References

Agostinho P, Cunha RA, Oliveira C. 2010. Neuroinflammation, oxidative stress and the pathogenesis of Alzheimer's disease. Curr Pharm Des 16(25):2766-78.

Aizenstein HJ, Nebes RD, Saxton JA, Price JC, Mathis CA, Tsopelas ND, Ziolko SK, James JA, Snitz BE, Houck PR and others. 2008. Frequent amyloid deposition without significant cognitive impairment among the elderly. Arch Neurol 65(11):1509-17.

Ajami B, Bennett JL, Krieger C, Tetzlaff W, Rossi FM. 2007. Local self-renewal can sustain CNS microglia maintenance and function throughout adult life. Nat Neurosci 10(12):1538-43.

Akiyama H, Barger S, Barnum S, Bradt B, Bauer J, Cole GM, Cooper NR, Eikelenboom P, Emmerling M, Fiebich BL and others. 2000. Inflammation and Alzheimer's disease. Neurobiol Aging 21(3):383-421.

Akiyama H, Kondo H, Ikeda K, Kato M, McGeer PL. 2001. Immunohistochemical localization of neprilysin in the human cerebral cortex: inverse association with vulnerability to amyloid beta-protein (Abeta) deposition. Brain Res 902(2):277-81.

Albert MS, DeKosky ST, Dickson D, Dubois B, Feldman HH, Fox NC, Gamst A, Holtzman DM, Jagust WJ, Petersen RC and others. 2011. The diagnosis of mild cognitive impairment due to Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. Alzheimers Dement 7(3):270-9.

Alexandrov PN, Dua P, Lukiw WJ. 2014. Up-Regulation of miRNA-146a in Progressive, Age-Related Inflammatory Neurodegenerative Disorders of the Human CNS. Front Neurol 5:181.

Allinson TM, Parkin ET, Turner AJ, Hooper NM. 2003. ADAMs family members as amyloid precursor protein alpha-secretases. J Neurosci Res 74(3):342-52.

Allsop D. 2000. Introduction to Alzheimer's disease. Methods Mol Med 32:1-21.

Aloisi F. 2001. Immune function of microglia. Glia 36(2):165-79.

Alves L, Correia AS, Miguel R, Alegria P, Bugalho P. 2012. Alzheimer's disease: a clinical practice-oriented review. Front Neurol 3:63.

Ames BN, Elson-Schwab I, Silver EA. 2002. High-dose vitamin therapy stimulates variant enzymes with decreased coenzyme binding affinity (increased K(m)): relevance to genetic disease and polymorphisms. Am J Clin Nutr 75(4):616-58.

An K, Klyubin I, Kim Y, Jung JH, Mably AJ, O'Dowd ST, Lynch T, Kanmert D, Lemere CA, Finan GM and others. 2013. Exosomes neutralize synaptic-plasticity-disrupting activity of Abeta assemblies in vivo. Mol Brain 6:47.

Anoop A, Singh PK, Jacob RS, Maji SK. 2010. CSF Biomarkers for Alzheimer's Disease Diagnosis. Int J Alzheimers Dis 2010.

Aston-Mourney K, Zraika S, Udayasankar J, Subramanian SL, Green PS, Kahn SE, Hull RL. 2013. Matrix metalloproteinase-9 reduces islet amyloid formation by degrading islet amyloid polypeptide. J Biol Chem 288(5):3553-9.

Austin SA, Santhanam AV, Katusic ZS. 2010. Endothelial nitric oxide modulates expression and processing of amyloid precursor protein. Circ Res 107(12):1498-502.

Bachstetter AD, Morganti JM, Jernberg J, Schlunk A, Mitchell SH, Brewster KW, Hudson CE, Cole MJ, Harrison JK, Bickford PC and others. 2011. Fractalkine and CX 3 CR1 regulate hippocampal neurogenesis in adult and aged rats. Neurobiol Aging 32(11):2030-44.

Bachstetter AD, Van Eldik LJ, Schmitt FA, Neltner JH, Ighodaro ET, Webster SJ, Patel E, Abner EL, Kryscio RJ, Nelson PT. 2015. Disease-related microglia heterogeneity in the hippocampus of Alzheimer's disease, dementia with Lewy bodies, and hippocampal sclerosis of aging. Acta Neuropathol Commun 3:32.

Backstrom JR, Lim GP, Cullen MJ, Tokes ZA. 1996. Matrix metalloproteinase-9 (MMP-9) is synthesized in neurons of the human hippocampus and is capable of degrading the amyloid-beta peptide (1-40). J Neurosci 16(24):7910-9.

Bader Lange ML, Cenini G, Piroddi M, Abdul HM, Sultana R, Galli F, Memo M, Butterfield DA. 2008. Loss of phospholipid asymmetry and elevated brain apoptotic protein levels in subjects with amnestic mild cognitive impairment and Alzheimer disease. Neurobiol Dis 29(3):456-64.

Bal-Price A, Brown GC. 2001. Inflammatory neurodegeneration mediated by nitric oxide from activated glia-inhibiting neuronal respiration, causing glutamate release and excitotoxicity. J Neurosci 21(17):6480-91.

Balducci C, Forloni G. 2011. APP transgenic mice: their use and limitations. Neuromolecular Med 13(2):117-37.

Bamberger ME, Harris ME, McDonald DR, Husemann J, Landreth GE. 2003. A cell surface receptor complex for fibrillar beta-amyloid mediates microglial activation. J Neurosci 23(7):2665-74.

Bard F, Cannon C, Barbour R, Burke RL, Games D, Grajeda H, Guido T, Hu K, Huang J, Johnson-Wood K and others. 2000. Peripherally administered antibodies against amyloid beta-peptide enter the central nervous system and reduce pathology in a mouse model of Alzheimer disease. Nat Med 6(8):916-9.

Barger SW. 2004. An unconventional hypothesis of oxidation in Alzheimer's disease: intersections with excitotoxicity. Front Biosci 9:3286-95.

Barger SW, Basile AS. 2001. Activation of microglia by secreted amyloid precursor protein evokes release of glutamate by cystine exchange and attenuates synaptic function. J Neurochem 76(3):846-54.

Bartel DP. 2009. MicroRNAs: target recognition and regulatory functions. Cell 136(2):215-33.

Bartzokis G, Sultzer D, Lu PH, Nuechterlein KH, Mintz J, Cummings JL. 2004. Heterogeneous age-related breakdown of white matter structural integrity: implications for cortical "disconnection" in aging and Alzheimer's disease. Neurobiol Aging 25(7):843-51.

Bayer TA, Wirths O. 2010. Intracellular accumulation of amyloid-Beta - a predictor for synaptic dysfunction and neuron loss in Alzheimer's disease. Front Aging Neurosci 2:8.

Becher B, Prat A, Antel JP. 2000. Brain-immune connection: immuno-regulatory properties of CNS-resident cells. Glia 29(4):293-304.

Bettens K, Sleegers K, Van Broeckhoven C. 2013. Genetic insights in Alzheimer's disease. Lancet Neurol 12(1):92-104.

Bhaskar K, Konerth M, Kokiko-Cochran ON, Cardona A, Ransohoff RM, Lamb BT. 2010. Regulation of tau pathology by the microglial fractalkine receptor. Neuron 68(1):19-31.

Bi X. 2010. Alzheimer disease: update on basic mechanisms. J Am Osteopath Assoc 110(9 Suppl 8):S3-9.

Bianchi ME, Manfredi AA. 2009. Immunology. Dangers in and out. Science 323(5922):1683-4.

Bianchi R, Kastrisianaki E, Giambanco I, Donato R. 2011. S100B protein stimulates microglia migration via RAGE-dependent up-regulation of chemokine expression and release. J Biol Chem 286(9):7214-26.

Biber K, Neumann H, Inoue K, Boddeke HW. 2007. Neuronal 'On' and 'Off' signals control microglia. Trends Neurosci 30(11):596-602.

Billings LM, Oddo S, Green KN, McGaugh JL, LaFerla FM. 2005. Intraneuronal Abeta causes the onset of early Alzheimer's disease-related cognitive deficits in transgenic mice. Neuron 45(5):675-88.

Bird L. 2011. Immune regulation: MicroRNAs keep microglia quiet. Nat Rev Immunol 11(2):76.

Block ML, Zecca L, Hong JS. 2007. Microglia-mediated neurotoxicity: uncovering the molecular mechanisms. Nat Rev Neurosci 8(1):57-69.

Bocchetta M, Galluzzi S, Kehoe PG, Aguera E, Bernabei R, Bullock R, Ceccaldi M, Dartigues JF, de Mendonca A, Didic M and others. 2015. The use of biomarkers for the etiologic diagnosis of MCI in Europe: an EADC survey. Alzheimers Dement 11(2):195-206 e1.

Boche D, Perry VH, Nicoll JA. 2013. Review: activation patterns of microglia and their identification in the human brain. Neuropathol Appl Neurobiol 39(1):3-18.

Bolmont T, Haiss F, Eicke D, Radde R, Mathis CA, Klunk WE, Kohsaka S, Jucker M, Calhoun ME. 2008. Dynamics of the microglial/amyloid interaction indicate a role in plaque maintenance. J Neurosci 28(16):4283-92.

Bonda DJ, Wang X, Perry G, Smith MA, Zhu X. 2010. Mitochondrial dynamics in Alzheimer's disease: opportunities for future treatment strategies. Drugs Aging 27(3):181-92.

Borchelt DR, Ratovitski T, van Lare J, Lee MK, Gonzales V, Jenkins NA, Copeland NG, Price DL, Sisodia SS. 1997. Accelerated amyloid deposition in the brains of transgenic mice coexpressing mutant presenilin 1 and amyloid precursor proteins. Neuron 19(4):939-45.

Bornemann KD, Wiederhold KH, Pauli C, Ermini F, Stalder M, Schnell L, Sommer B, Jucker M, Staufenbiel M. 2001. Abeta-induced inflammatory processes in microglia cells of APP23 transgenic mice. Am J Pathol 158(1):63-73.

Bose S, Kim S, Oh Y, Moniruzzaman M, Lee G, Cho J. 2016. Effect of CCL2 on BV2 microglial cell migration: Involvement of probable signaling pathways. Cytokine 81:39-49.

Braak H, Braak E. 1991. Neuropathological Staging of Alzheimer-Related Changes. Acta Neuropathologica 82(4):239-259.

Braak H, Braak E. 1996. Development of Alzheimer-related neurofibrillary changes in the neocortex inversely recapitulates cortical myelogenesis. Acta Neuropathol 92(2):197-201.

Braak H, Del Tredici K. 2011. The pathological process underlying Alzheimer's disease in individuals under thirty. Acta Neuropathol 121(2):171-81.

Braak H, Del Tredici K, Schultz C, Braak E. 2000. Vulnerability of select neuronal types to Alzheimer's disease. Ann N Y Acad Sci 924:53-61.

Bredesen DE. 2016. Inhalational Alzheimer's disease: an unrecognized - and treatable - epidemic. Aging (Albany NY).

Brewer GJ. 1997. Isolation and culture of adult rat hippocampal neurons. J Neurosci Methods 71(2):143-55.

Brites D, C. G, R. P, A.S. F. 2015. New insights in the pro- and anti-neurogenic interventions of microglia and their association to gliomas. In: E.R.Giffard, editor. Physiology, Regulation and Health Implications. New York: Nova Biomedical. p 67-111.

Brites D, Fernandes A. 2015. Neuroinflammation and Depression: Microglia Activation, Extracellular Microvesicles and microRNA Dysregulation. Front Cell Neurosci 9:476.

Brites D, Vaz AR. 2014. Microglia centered pathogenesis in ALS: insights in cell interconnectivity. Front Cell Neurosci 8:117.

Butovsky O, Jedrychowski MP, Cialic R, Krasemann S, Murugaiyan G, Fanek Z, Greco DJ, Wu PM, Doykan CE, Kiner O and others. 2015. Targeting miR-155 restores abnormal microglia and attenuates disease in SOD1 mice. Ann Neurol 77(1):75-99.

Cacabelos R, Torrellas C, Carrera I, Cacabelos P, Corzo L, Fernandez-Novoa L, Tellado I, Carril JC, Aliev G. 2016. Novel Therapeutic Strategies for Dementia. CNS Neurol Disord Drug Targets.

Caccamo A, Oddo S, Sugarman MC, Akbari Y, LaFerla FM. 2005. Age- and region-dependent alterations in Abeta-degrading enzymes: implications for Abeta-induced disorders. Neurobiol Aging 26(5):645-54.

Cagnin A, Brooks DJ, Kennedy AM, Gunn RN, Myers R, Turkheimer FE, Jones T, Banati RB. 2001. In-vivo measurement of activated microglia in dementia. Lancet 358(9280):461-7.

Calvo M, Bennett DL. 2011. The mechanisms of microgliosis and pain following peripheral nerve injury. Exp Neurol 234(2):271-82.

Cameron B, Landreth GE. 2010. Inflammation, microglia, and Alzheimer's disease. Neurobiol Dis 37(3):503-9.

Cardona AE, Pioro EP, Sasse ME, Kostenko V, Cardona SM, Dijkstra IM, Huang D, Kidd G, Dombrowski S, Dutta R and others. 2006. Control of microglial neurotoxicity by the fractalkine receptor. Nat Neurosci 9(7):917-24.

Cardoso AL, Guedes JR, Pereira de Almeida L, Pedroso de Lima MC. 2012. miR-155 modulates microglia-mediated immune response by down-regulating SOCS-1 and promoting cytokine and nitric oxide production. Immunology 135(1):73-88.

Carreira BP, Morte MI, Carvalho CM, Araujo IM. 2012. Assessing the influence of neuroinflammation on neurogenesis: in vitro models using neural stem cells and microglia as valuable research tools. In: Sun T, editor. Neural stem cells and therapy: InTech.

Caruso D, Barron AM, Brown MA, Abbiati F, Carrero P, Pike CJ, Garcia-Segura LM, Melcangi RC. 2013. Age-related changes in neuroactive steroid levels in 3xTg-AD mice. Neurobiol Aging 34(4):1080-9.

Cassano T, Pace L, Bedse G, Lavecchia AM, De Marco F, Gaetani S, Serviddio G. 2016. Glutamate and Mitochondria: Two Prominent Players in the Oxidative Stress-Induced Neurodegeneration. Curr Alzheimer Res 13(2):185-97.

Cavallucci V, D'Amelio M, Cecconi F. 2012. Abeta toxicity in Alzheimer's disease. Mol Neurobiol 45(2):366-78.

Cervellati C, Wood PL, Romani A, Valacchi G, Squerzanti M, Sanz JM, Ortolani B, Zuliani G. 2016. Oxidative challenge in Alzheimer's disease: state of knowledge and future needs. J Investig Med 64(1):21-32.

Chakrabarty P, Tianbai L, Herring A, Ceballos-Diaz C, Das P, Golde TE. 2012. Hippocampal expression of murine IL-4 results in exacerbation of amyloid deposition. Mol Neurodegener 7:36.

Chao CC, Molitor TW, Hu S. 1993. Neuroprotective role of IL-4 against activated microglia. J Immunol 151(3):1473-81.

Chasseigneaux S, Allinquant B. 2012. Functions of Abeta, sAPPalpha and sAPPbeta : similarities and differences. J Neurochem 120 Suppl 1:99-108.

Chen K, Iribarren P, Hu J, Chen J, Gong W, Cho EH, Lockett S, Dunlop NM, Wang JM. 2006. Activation of Toll-like receptor 2 on microglia promotes cell uptake of Alzheimer disease-associated amyloid beta peptide. J Biol Chem 281(6):3651-9.

Chen Z, Jalabi W, Hu W, Park HJ, Gale JT, Kidd GJ, Bernatowicz R, Gossman ZC, Chen JT, Dutta R and others. 2014. Microglial displacement of inhibitory synapses provides neuroprotection in the adult brain. Nat Commun 5:4486.

Cheng L, Doecke JD, Sharples RA, Villemagne VL, Fowler CJ, Rembach A, Martins RN, Rowe CC, Macaulay SL, Masters CL and others. 2014a. Prognostic serum miRNA biomarkers associated with Alzheimer's disease shows concordance with neuropsychological and neuroimaging assessment. Mol Psychiatry 20(10):1188-96.

Cheng L, Sharples RA, Scicluna BJ, Hill AF. 2014b. Exosomes provide a protective and enriched source of miRNA for biomarker profiling compared to intracellular and cell-free blood. J Extracell Vesicles 3.

Cheng L, Sun X, Scicluna BJ, Coleman BM, Hill AF. 2013. Characterization and deep sequencing analysis of exosomal and non-exosomal miRNA in human urine. Kidney Int 86(2):433-44.

Cherry JD, Olschowka JA, O'Banion MK. 2014. Neuroinflammation and M2 microglia: the good, the bad, and the inflamed. J Neuroinflammation 11:98.

Chhor V, Le Charpentier T, Lebon S, Ore MV, Celador IL, Josserand J, Degos V, Jacotot E, Hagberg H, Savman K and others. 2013. Characterization of phenotype markers and neuronotoxic potential of polarised primary microglia in vitro. Brain Behav Immun 32:70-85.

Choi SH, Kim YH, Hebisch M, Sliwinski C, Lee S, D'Avanzo C, Chen H, Hooli B, Asselin C, Muffat J and others. 2014. A three-dimensional human neural cell culture model of Alzheimer's disease. Nature 515(7526):274-8.

Choi SH, Kim YH, Quinti L, Tanzi RE, Kim DY. 2016. 3D culture models of Alzheimer's disease: a road map to a "cure-in-a-dish". Mol Neurodegener 11(1):75.

Chromy BA, Nowak RJ, Lambert MP, Viola KL, Chang L, Velasco PT, Jones BW, Fernandez SJ, Lacor PN, Horowitz P and others. 2003. Self-assembly of Abeta(1-42) into globular neurotoxins. Biochemistry 42(44):12749-60.

Ciryam P, Kundra R, Freer R, Morimoto RI, Dobson CM, Vendruscolo M. 2016. A transcriptional signature of Alzheimer's disease is associated with a metastable subproteome at risk for aggregation. Proc Natl Acad Sci U S A 113(17):4753-8.

Clark AK, Gruber-Schoffnegger D, Drdla-Schutting R, Gerhold KJ, Malcangio M, Sandkuhler J. 2015. Selective activation of microglia facilitates synaptic strength. J Neurosci 35(11):4552-70.

Clark AK, Yip PK, Grist J, Gentry C, Staniland AA, Marchand F, Dehvari M, Wotherspoon G, Winter J, Ullah J and others. 2007. Inhibition of spinal microglial cathepsin S for the reversal of neuropathic pain. Proc Natl Acad Sci U S A 104(25):10655-60.

Cohen AD, Rabinovici GD, Mathis CA, Jagust WJ, Klunk WE, Ikonomovic MD. 2012. Using Pittsburgh Compound B for in vivo PET imaging of fibrillar amyloid-beta. Adv Pharmacol 64:27-81.

Colton CA. 2009. Heterogeneity of microglial activation in the innate immune response in the brain. J Neuroimmune Pharmacol 4(4):399-418.

Colton CA, Mott RT, Sharpe H, Xu Q, Van Nostrand WE, Vitek MP. 2006. Expression profiles for macrophage alternative activation genes in AD and in mouse models of AD. J Neuroinflammation 3:27.

Combs CK, Karlo JC, Kao SC, Landreth GE. 2001. beta-Amyloid stimulation of microglia and monocytes results in TNFalpha-dependent expression of inducible nitric oxide synthase and neuronal apoptosis. J Neurosci 21(4):1179-88.

Conde JR, Streit WJ. 2006. Microglia in the aging brain. J Neuropathol Exp Neurol 65(3):199-203.

Costello DA, Lyons A, Denieffe S, Browne TC, Cox FF, Lynch MA. 2011. Long term potentiation is impaired in membrane glycoprotein CD200-deficient mice: a role for Toll-like receptor activation. J Biol Chem 286(40):34722-32.

Crespo AC, Silva B, Marques L, Marcelino E, Maruta C, Costa S, Timoteo A, Vilares A, Couto FS, Faustino P and others. 2014. Genetic and biochemical markers in patients with Alzheimer's disease support a concerted systemic iron homeostasis dysregulation. Neurobiol Aging 35(4):777-85.

Crews L, Masliah E. 2010. Molecular mechanisms of neurodegeneration in Alzheimer's disease. Hum Mol Genet 19(R1):R12-20.

Cristovão AC, Saavedra A, Fonseca CP, Campos F, Duarte EP, Baltazar G. 2010. Microglia of rat ventral midbrain recovers its resting state over time in vitro: let microglia rest before work. J Neurosci Res 88(3):552-62.

Cui JG, Li YY, Zhao Y, Bhattacharjee S, Lukiw WJ. 2010. Differential regulation of interleukin-1 receptor-associated kinase-1 (IRAK-1) and IRAK-2 by microRNA-146a and NF-kappaB in stressed human astroglial cells and in Alzheimer disease. J Biol Chem 285(50):38951-60.

Culbert AA, Skaper SD, Howlett DR, Evans NA, Facci L, Soden PE, Seymour ZM, Guillot F, Gaestel M, Richardson JC. 2006. MAPK-activated protein kinase 2 deficiency in microglia inhibits pro-inflammatory mediator release and resultant

neurotoxicity. Relevance to neuroinflammation in a transgenic mouse model of Alzheimer disease. J Biol Chem 281(33):23658-67.
Czeh M, Gressens P, Kaindl AM. 2011. The yin and yang of microglia. Dev Neurosci 33(3-4):199-209.
Damani MR, Zhao L, Fontainhas AM, Amaral J, Fariss RN, Wong WT. 2011. Age-related alterations in the dynamic behavior of microglia. Aging Cell 10(2):263-76.
Davalos D, Grutzendler J, Yang G, Kim JV, Zuo Y, Jung S, Littman DR, Dustin ML, Gan WB. 2005. ATP mediates rapid microglial response to local brain injury in vivo. Nat Neurosci 8(6):752-8.
De Simone R, Ajmone-Cat MA, Minghetti L. 2004. Atypical antiinflammatory activation of microglia induced by apoptotic neurons: possible role of phosphatidylserine-phosphatidylserine receptor interaction. Mol Neurobiol 29(2):197-212.
del Rio-Hortega P. 1993. Art and artifice in the science of histology. 1933. Histopathology 22(6):515-25.
Demarin V, Kes VB, Morovic S, Zavoreo I. 2009. Evaluation of aging vs dementia by means of neurosonology. J Neurol Sci 283(1-2):9-12.
Demarin V, Zavoreo I, Kes VB, Simundic AM. 2011. Biomarkers in Alzheimer's disease. Clin Chem Lab Med 49(5):773-8.
Dhawan G, Combs CK. 2012. Inhibition of Src kinase activity attenuates amyloid associated microgliosis in a murine model of Alzheimer's disease. J Neuroinflammation 9:117.
Dinkins MB, Dasgupta S, Wang G, Zhu G, Bieberich E. 2014. Exosome reduction in vivo is associated with lower amyloid plaque load in the 5XFAD mouse model of Alzheimer's disease. Neurobiol Aging 35(8):1792-800.
Do Carmo S, Cuello AC. 2013. Modeling Alzheimer's disease in transgenic rats. Mol Neurodegener 8:37.
Doi Y, Mizuno T, Maki Y, Jin S, Mizoguchi H, Ikeyama M, Doi M, Michikawa M, Takeuchi H, Suzumura A. 2009. Microglia activated with the toll-like receptor 9 ligand CpG attenuate oligomeric amyloid {beta} neurotoxicity in in vitro and in vivo models of Alzheimer's disease. Am J Pathol 175(5):2121-32.
Dou Y, Wu HJ, Li HQ, Qin S, Wang YE, Li J, Lou HF, Chen Z, Li XM, Luo QM and others. 2012. Microglial migration mediated by ATP-induced ATP release from lysosomes. Cell Res 22(6):1022-33.
Duan Y, Dong S, Gu F, Hu Y, Zhao Z. 2012. Advances in the pathogenesis of Alzheimer's disease: focusing on tau-mediated neurodegeneration. Transl Neurodegener 1(1):24.
Durafourt BA, Moore CS, Zammit DA, Johnson TA, Zaguia F, Guiot MC, Bar-Or A, Antel JP. 2012. Comparison of polarization properties of human adult microglia and blood-derived macrophages. Glia 60(5):717-27.
Duyckaerts C, Potier MC, Delatour B. 2008. Alzheimer disease models and human neuropathology: similarities and differences. Acta Neuropathol 115(1):5-38.
Echeverria V, Yarkov A, Aliev G. 2016. Positive modulators of the alpha7 nicotinic receptor against neuroinflammation and cognitive impairment in Alzheimer's disease. Prog Neurobiol.
Eikelenboom P, van Exel E, Hoozemans JJ, Veerhuis R, Rozemuller AJ, van Gool WA. 2010. Neuroinflammation - an early event in both the history and pathogenesis of Alzheimer's disease. Neurodegener Dis 7(1-3):38-41.
Eimer WA, Vassar R. 2013. Neuron loss in the 5XFAD mouse model of Alzheimer's disease correlates with intraneuronal Abeta42 accumulation and Caspase-3 activation. Mol Neurodegener 8:2.
El Khoury J, Luster AD. 2008. Mechanisms of microglia accumulation in Alzheimer's disease: therapeutic implications. Trends Pharmacol Sci 29(12):626-32.
El Khoury J, Toft M, Hickman SE, Means TK, Terada K, Geula C, Luster AD. 2007. Ccr2 deficiency impairs microglial accumulation and accelerates progression of Alzheimer-like disease. Nat Med 13(4):432-8.

El Khoury JB, Moore KJ, Means TK, Leung J, Terada K, Toft M, Freeman MW, Luster AD. 2003. CD36 mediates the innate host response to beta-amyloid. J Exp Med 197(12):1657-66.

Elder GA, Gama Sosa MA, De Gasperi R. 2010. Transgenic mouse models of Alzheimer's disease. Mt Sinai J Med 77(1):69-81.

Elliott MR, Chekeni FB, Trampont PC, Lazarowski ER, Kadl A, Walk SF, Park D, Woodson RI, Ostankovich M, Sharma P and others. 2009. Nucleotides released by apoptotic cells act as a find-me signal to promote phagocytic clearance. Nature 461(7261):282-6.

Elton TS, Selemon H, Elton SM, Parinandi NL. 2013. Regulation of the MIR155 host gene in physiological and pathological processes. Gene 532(1):1-12.

Eng LF, Ghirnikar RS, Lee YL. 2000. Glial fibrillary acidic protein: GFAP-thirty-one years (1969-2000). Neurochem Res 25(9-10):1439-51.

Enokido Y, Yoshitake A, Ito H, Okazawa H. 2008. Age-dependent change of HMGB1 and DNA double-strand break accumulation in mouse brain. Biochem Biophys Res Commun 376(1):128-33.

Falcão AS, Fernandes A, Brito MA, Silva RF, Brites D. 2006. Bilirubin-induced immunostimulant effects and toxicity vary with neural cell type and maturation state. Acta Neuropathol 112(1):95-105.

Fandrich M, Schmidt M, Grigorieff N. 2011. Recent progress in understanding Alzheimer's beta-amyloid structures. Trends Biochem Sci 36(6):338-45.

Fang P, Schachner M, Shen YQ. 2012. HMGB1 in development and diseases of the central nervous system. Mol Neurobiol 45(3):499-506.

Fenn AM, Henry CJ, Huang Y, Dugan A, Godbout JP. 2012. Lipopolysaccharide-induced interleukin (IL)-4 receptor-alpha expression and corresponding sensitivity to the M2 promoting effects of IL-4 are impaired in microglia of aged mice. Brain Behav Immun 26(5):766-77.

Fiandaca MS, Kapogiannis D, Mapstone M, Boxer A, Eitan E, Schwartz JB, Abner EL, Petersen RC, Federoff HJ, Miller BL and others. 2014. Identification of preclinical Alzheimer's disease by a profile of pathogenic proteins in neurally derived blood exosomes: A case-control study. Alzheimers Dement 11(6):600-7 e1.

Flanary BE, Sammons NW, Nguyen C, Walker D, Streit WJ. 2007. Evidence that aging and amyloid promote microglial cell senescence. Rejuvenation Res 10(1):61-74.

Flanary BE, Streit WJ. 2003. Telomeres shorten with age in rat cerebellum and cortex in vivo. J Anti Aging Med 6(4):299-308.

Flanary BE, Streit WJ. 2004. Progressive telomere shortening occurs in cultured rat microglia, but not astrocytes. Glia 45(1):75-88.

Fletcher LC, Burke KE, Caine PL, Rinne NL, Braniff CA, Davis HR, Miles KA, Packer C. 2013. Diagnosing Alzheimer's disease: are we any nearer to useful biomarker-based, non-invasive tests? GMS Health Technol Assess 9:Doc01.

Floden AM, Combs CK. 2011. Microglia demonstrate age-dependent interaction with amyloid-beta fibrils. J Alzheimers Dis 25(2):279-93.

Foley AM, Ammar ZM, Lee RH, Mitchell CS. 2015. Systematic review of the relationship between amyloid-beta levels and measures of transgenic mouse cognitive deficit in Alzheimer's disease. J Alzheimers Dis 44(3):787-95.

Franciosi S, Choi HB, Kim SU, McLarnon JG. 2005. IL-8 enhancement of amyloid-beta (Abeta 1-42)-induced expression and production of pro-inflammatory cytokines and COX-2 in cultured human microglia. J Neuroimmunol 159(1-2):66-74.

Fratiglioni L, Qiu C. 2009. Prevention of common neurodegenerative disorders in the elderly. Exp Gerontol 44(1-2):46-50.

Frautschy SA, Yang F, Irrizarry M, Hyman B, Saido TC, Hsiao K, Cole GM. 1998. Microglial response to amyloid plaques in APPsw transgenic mice. Am J Pathol 152(1):307-17.

Freilich RW, Woodbury ME, Ikezu T. 2013. Integrated expression profiles of mRNA and miRNA in polarized primary murine microglia. PLoS One 8(11):e79416.

Fuhrmann M, Bittner T, Jung CK, Burgold S, Page RM, Mitteregger G, Haass C, LaFerla FM, Kretzschmar H, Herms J. 2010. Microglial Cx3cr1 knockout prevents neuron loss in a mouse model of Alzheimer's disease. Nat Neurosci 13(4):411-3.

Fuhrmann M, Ochs S, Keppler K, Langer A, Hoelter S, Herms J. 2011. Role of chemokine receptor CX3CR1 in Alzheimer's transgenic mice. Alzheimer's & Dementia: The Journal of the Alzheimer's Association 7(4):S564.

Fuller AD, Van Eldik LJ. 2008. MFG-E8 regulates microglial phagocytosis of apoptotic neurons. J Neuroimmune Pharmacol 3(4):246-56.

Gallo A, Tandon M, Alevizos I, Illei GG. 2012. The majority of microRNAs detectable in serum and saliva is concentrated in exosomes. PLoS One 7(3):e30679.

Gemma C, Bachstetter AD. 2013. The role of microglia in adult hippocampal neurogenesis. Front Cell Neurosci 7:229.

Gimenez-Llort L, Blazquez G, Canete T, Johansson B, Oddo S, Tobena A, LaFerla FM, Fernandez-Teruel A. 2007. Modeling behavioral and neuronal symptoms of Alzheimer's disease in mice: a role for intraneuronal amyloid. Neurosci Biobehav Rev 31(1):125-47.

Ginhoux F, Lim S, Hoeffel G, Low D, Huber T. 2013. Origin and differentiation of microglia. Front Cell Neurosci 7:45.

Glass CK, Saijo K, Winner B, Marchetto MC, Gage FH. 2010. Mechanisms underlying inflammation in neurodegeneration. Cell 140(6):918-34.

Gomez-Nicola D, Perry VH. 2015. Microglial dynamics and role in the healthy and diseased brain: a paradigm of functional plasticity. Neuroscientist 21(2):169-84.

Gomez-Ramos A, Diaz-Hernandez M, Cuadros R, Hernandez F, Avila J. 2006. Extracellular tau is toxic to neuronal cells. FEBS Lett 580(20):4842-50.

Gong CX, Grundke-Iqbal I, Iqbal K. 2010. Targeting tau protein in Alzheimer's disease. Drugs Aging 27(5):351-65.

Gouras GK, Almeida CG, Takahashi RH. 2005. Intraneuronal Abeta accumulation and origin of plaques in Alzheimer's disease. Neurobiol Aging 26(9):1235-44.

Gouras GK, Tampellini D, Takahashi RH, Capetillo-Zarate E. 2010. Intraneuronal beta-amyloid accumulation and synapse pathology in Alzheimer's disease. Acta Neuropathol 119(5):523-41.

Gouras GK, Tsai J, Naslund J, Vincent B, Edgar M, Checler F, Greenfield JP, Haroutunian V, Buxbaum JD, Xu H and others. 2000. Intraneuronal Abeta42 accumulation in human brain. Am J Pathol 156(1):15-20.

Graeber MB. 2010. Changing face of microglia. Science 330(6005):783-8.

Graeber MB, Streit WJ. 2010. Microglia: biology and pathology. Acta Neuropathol 119(1):89-105.

Grammas P, Tripathy D, Sanchez A, Yin X, Luo J. 2011. Brain microvasculature and hypoxia-related proteins in Alzheimer's disease. Int J Clin Exp Pathol 4(6):616-27.

Gresa-Arribas N, Vieitez C, Dentesano G, Serratosa J, Saura J, Sola C. 2012. Modelling neuroinflammation in vitro: a tool to test the potential neuroprotective effect of anti-inflammatory agents. PLoS One 7(9):e45227.

Greter M, Merad M. 2013. Regulation of microglia development and homeostasis. Glia 61(1):121-7.

Guedes J, Cardoso AL, Pedroso de Lima MC. 2013. Involvement of microRNA in microglia-mediated immune response. Clin Dev Immunol 2013:186872.

Guedes JR, Custodia CM, Silva RJ, de Almeida LP, Pedroso de Lima MC, Cardoso AL. 2014. Early miR-155 upregulation contributes to neuroinflammation in Alzheimer's disease triple transgenic mouse model. Hum Mol Genet 23(23):6286-301.

Guerreiro C, Silva B, Crespo AC, Marques L, Costa S, Timoteo A, Marcelino E, Maruta C, Vilares A, Matos M and others. 2015. Decrease in APP and CP mRNA expression supports impairment of iron export in Alzheimer's disease patients. Biochim Biophys Acta 1852(10 Pt A):2116-22.

Guix FX, Uribesalgo I, Coma M, Munoz FJ. 2005. The physiology and pathophysiology of nitric oxide in the brain. Prog Neurobiol 76(2):126-52.

Gyoneva S, Orr AG, Traynelis SF. 2009. Differential regulation of microglial motility by ATP/ADP and adenosine. Parkinsonism Relat Disord 15 Suppl 3:S195-9.

Hanayama R, Tanaka M, Miwa K, Shinohara A, Iwamatsu A, Nagata S. 2002. Identification of a factor that links apoptotic cells to phagocytes. Nature 417(6885):182-7.

Hanisch UK, Kettenmann H. 2007. Microglia: active sensor and versatile effector cells in the normal and pathologic brain. Nat Neurosci 10(11):1387-94.

Hardy J, Selkoe DJ. 2002. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. Science 297(5580):353-6.

Harman D. 2006. Alzheimer's disease pathogenesis: role of aging. Ann N Y Acad Sci 1067:454-60.

Haynes SE, Hollopeter G, Yang G, Kurpius D, Dailey ME, Gan WB, Julius D. 2006. The P2Y12 receptor regulates microglial activation by extracellular nucleotides. Nat Neurosci 9(12):1512-9.

Hebert LE, Scherr PA, Bienias JL, Bennett DA, Evans DA. 2003. Alzheimer disease in the US population: prevalence estimates using the 2000 census. Arch Neurol 60(8):1119-22.

Heneka MT, Carson MJ, El Khoury J, Landreth GE, Brosseron F, Feinstein DL, Jacobs AH, Wyss-Coray T, Vitorica J, Ransohoff RM and others. 2015. Neuroinflammation in Alzheimer's disease. Lancet Neurol 14(4):388-405.

Heneka MT, Kummer MP, Stutz A, Delekate A, Schwartz S, Vieira-Saecker A, Griep A, Axt D, Remus A, Tzeng TC and others. 2013. NLRP3 is activated in Alzheimer's disease and contributes to pathology in APP/PS1 mice. Nature 493(7434):674-8.

Heneka MT, O'Banion MK, Terwel D, Kummer MP. 2010. Neuroinflammatory processes in Alzheimer's disease. J Neural Transm (Vienna) 117(8):919-47.

Herber DL, Mercer M, Roth LM, Symmonds K, Maloney J, Wilson N, Freeman MJ, Morgan D, Gordon MN. 2007. Microglial activation is required for Abeta clearance after intracranial injection of lipopolysaccharide in APP transgenic mice. J Neuroimmune Pharmacol 2(2):222-31.

Hickman SE, Allison EK, El Khoury J. 2008. Microglial dysfunction and defective beta-amyloid clearance pathways in aging Alzheimer's disease mice. J Neurosci 28(33):8354-60.

Hoarau JJ, Krejbich-Trotot P, Jaffar-Bandjee MC, Das T, Thon-Hon GV, Kumar S, Neal JW, Gasque P. 2011. Activation and control of CNS innate immune responses in health and diseases: a balancing act finely tuned by neuroimmune regulators (NIReg). CNS Neurol Disord Drug Targets 10(1):25-43.

Holcomb L, Gordon MN, McGowan E, Yu X, Benkovic S, Jantzen P, Wright K, Saad I, Mueller R, Morgan D and others. 1998. Accelerated Alzheimer-type phenotype in transgenic mice carrying both mutant amyloid precursor protein and presenilin 1 transgenes. Nat Med 4(1):97-100.

Horiuchi M, Maezawa I, Itoh A, Wakayama K, Jin LW, Itoh T, Decarli C. 2010. Amyloid beta1-42 oligomer inhibits myelin sheet formation in vitro. Neurobiol Aging 33(3):499-509.

Horvath RJ, Nutile-McMenemy N, Alkaitis MS, Deleo JA. 2008. Differential migration, LPS-induced cytokine, chemokine, and NO expression in immortalized BV-2 and HAPI cell lines and primary microglial cultures. J Neurochem 107(2):557-69.

Howlett DR, Richardson JC. 2009. The pathology of APP transgenic mice: a model of Alzheimer's disease or simply overexpression of APP? Histol Histopathol 24(1):83-100.

Huang Y, Mucke L. 2012. Alzheimer mechanisms and therapeutic strategies. Cell 148(6):1204-22.

Hughes V. 2012. Microglia: The constant gardeners. Nature 485(7400):570-2.

Humpel C. 2015. Organotypic brain slice cultures: A review. Neuroscience 305:86-98.
Huynh TV, Davis AA, Ulrich JD, Holtzman DM. 2017. Apolipoprotein E and Alzheimer Disease: The influence of apoE on amyloid-beta and other amyloidogenic proteins. J Lipid Res.
Ihara M, Tomimoto H, Kinoshita M, Oh J, Noda M, Wakita H, Akiguchi I, Shibasaki H. 2001. Chronic cerebral hypoperfusion induces MMP-2 but not MMP-9 expression in the microglia and vascular endothelium of white matter. J Cereb Blood Flow Metab 21(7):828-34.
Ihara R. 2016. [New diagnostic guidelines for Alzheimer's disease by NIA/AA]. Nihon Rinsho 74(3):386-94.
Inoue K. 2006. The function of microglia through purinergic receptors: neuropathic pain and cytokine release. Pharmacol Ther 109(1-2):210-26.
Isaev NK, Stelmashook EV, Genrikhs EE, Oborina MV, Kapkaeva MR, Skulachev VP. 2015. Alzheimer's Disease: An Exacerbation of Senile Phenoptosis. Biochemistry (Mosc) 80(12):1578-81.
Isik AT. 2010. Late onset Alzheimer's disease in older people. Clin Interv Aging 5:307-11.
Ito D, Tanaka K, Suzuki S, Dembo T, Fukuuchi Y. 2001. Enhanced expression of Iba1, ionized calcium-binding adapter molecule 1, after transient focal cerebral ischemia in rat brain. Stroke 32(5):1208-15.
Ito S, Kimura K, Haneda M, Ishida Y, Sawada M, Isobe K. 2007. Induction of matrix metalloproteinases (MMP3, MMP12 and MMP13) expression in the microglia by amyloid-beta stimulation via the PI3K/Akt pathway. Exp Gerontol 42(6):532-7.
Jack CR, Jr., Albert MS, Knopman DS, McKhann GM, Sperling RA, Carrillo MC, Thies B, Phelps CH. 2011. Introduction to the recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. Alzheimers Dement 7(3):257-62.
Jack CR, Jr., Holtzman DM. 2013. Biomarker modeling of Alzheimer's disease. Neuron 80(6):1347-58.
Jana M, Palencia CA, Pahan K. 2008. Fibrillar amyloid-beta peptides activate microglia via TLR2: implications for Alzheimer's disease. J Immunol 181(10):7254-62.
Janelsins MC, Mastrangelo MA, Park KM, Sudol KL, Narrow WC, Oddo S, LaFerla FM, Callahan LM, Federoff HJ, Bowers WJ. 2008. Chronic neuron-specific tumor necrosis factor-alpha expression enhances the local inflammatory environment ultimately leading to neuronal death in 3xTg-AD mice. Am J Pathol 173(6):1768-82.
Jankowsky JL, Fadale DJ, Anderson J, Xu GM, Gonzales V, Jenkins NA, Copeland NG, Lee MK, Younkin LH, Wagner SL and others. 2004. Mutant presenilins specifically elevate the levels of the 42 residue beta-amyloid peptide in vivo: evidence for augmentation of a 42-specific gamma secretase. Hum Mol Genet 13(2):159-70.
Jankowsky JL, Slunt HH, Ratovitski T, Jenkins NA, Copeland NG, Borchelt DR. 2001. Co-expression of multiple transgenes in mouse CNS: a comparison of strategies. Biomol Eng 17(6):157-65.
Janssen B, Vugts DJ, Funke U, Molenaar GT, Kruijer PS, van Berckel BN, Lammertsma AA, Windhorst AD. 2015. Imaging of neuroinflammation in Alzheimer's disease, multiple sclerosis and stroke: Recent developments in positron emission tomography. Biochim Biophys Acta.
Jayadev S, Case A, Alajajian B, Eastman AJ, Moller T, Garden GA. 2013. Presenilin 2 influences miR146 level and activity in microglia. J Neurochem 127(5):592-9.
Ji K, Akgul G, Wollmuth LP, Tsirka SE. 2013. Microglia actively regulate the number of functional synapses. PLoS One 8(2):e56293.
Jiang M, Xiang Y, Wang D, Gao J, Liu D, Liu Y, Liu S, Zheng D. 2012a. Dysregulated expression of miR-146a contributes to age-related dysfunction of macrophages. Aging Cell 11(1):29-40.

Jiang T, Yu JT, Tan L. 2012b. Novel disease-modifying therapies for Alzheimer's disease. J Alzheimers Dis 31(3):475-92.

Jimenez S, Baglietto-Vargas D, Caballero C, Moreno-Gonzalez I, Torres M, Sanchez-Varo R, Ruano D, Vizuete M, Gutierrez A, Vitorica J. 2008. Inflammatory response in the hippocampus of PS1M146L/APP751SL mouse model of Alzheimer's disease: age-dependent switch in the microglial phenotype from alternative to classic. J Neurosci 28(45):11650-61.

Johnstone DM, Moro C, Stone J, Benabid AL, Mitrofanis J. 2016. Turning On Lights to Stop Neurodegeneration: The Potential of Near Infrared Light Therapy in Alzheimer's and Parkinson's Disease. Front Neurosci 9:500.

Kawabata H, Setoguchi T, Yone K, Souda M, Yoshida H, Kawahara K, Maruyama I, Komiya S. 2010. High mobility group box 1 is upregulated after spinal cord injury and is associated with neuronal cell apoptosis. Spine (Phila Pa 1976) 35(11):1109-15.

Kettenmann H, Hanisch UK, Noda M, Verkhratsky A. 2011. Physiology of microglia. Physiol Rev 91(2):461-553.

Kettenmann H, Kirchhoff F, Verkhratsky A. 2013. Microglia: new roles for the synaptic stripper. Neuron 77(1):10-8.

Khairallah MI, Kassem LA. 2011. Alzheimer's disease: current status of etiopathogenesis and therapeutic strategies. Pak J Biol Sci 14(4):257-72.

Kidd PM. 2008. Alzheimer's disease, amnestic mild cognitive impairment, and age-associated memory impairment: current understanding and progress toward integrative prevention. Altern Med Rev 13(2):85-115.

Kierdorf K, Prinz M. 2013. Factors regulating microglia activation. Front Cell Neurosci 7:44.

Kim GH, Kim JE, Rhie SJ, Yoon S. 2015. The Role of Oxidative Stress in Neurodegenerative Diseases. Exp Neurobiol 24(4):325-40.

Kim HJ, Ajit D, Peterson TS, Wang Y, Camden JM, Gibson Wood W, Sun GY, Erb L, Petris M, Weisman GA. 2012. Nucleotides released from Abeta(1)(-)(4)(2) -treated microglial cells increase cell migration and Abeta(1)(-)(4)(2) uptake through P2Y(2) receptor activation. J Neurochem 121(2):228-38.

Kim J, Basak JM, Holtzman DM. 2009. The role of apolipoprotein E in Alzheimer's disease. Neuron 63(3):287-303.

Kim SU, de Vellis J. 2005. Microglia in health and disease. J Neurosci Res 81(3):302-13.

Kim SY, Moon JH, Lee HG, Kim SU, Lee YB. 2007. ATP released from beta-amyloid-stimulated microglia induces reactive oxygen species production in an autocrine fashion. Exp Mol Med 39(6):820-7.

Kim T, Vidal GS, Djurisic M, William CM, Birnbaum ME, Garcia KC, Hyman BT, Shatz CJ. 2013. Human LilrB2 is a beta-amyloid receptor and its murine homolog PirB regulates synaptic plasticity in an Alzheimer's model. Science 341(6152):1399-404.

Kiyota T, Okuyama S, Swan RJ, Jacobsen MT, Gendelman HE, Ikezu T. 2010. CNS expression of anti-inflammatory cytokine interleukin-4 attenuates Alzheimer's disease-like pathogenesis in APP+PS1 bigenic mice. FASEB J 24(8):3093-102.

Kobayashi K, Hayashi M, Nakano H, Fukutani Y, Sasaki K, Shimazaki M, Koshino Y. 2002. Apoptosis of astrocytes with enhanced lysosomal activity and oligodendrocytes in white matter lesions in Alzheimer's disease. Neuropathol Appl Neurobiol 28(3):238-51.

Koenigsknecht-Talboo J, Landreth GE. 2005. Microglial phagocytosis induced by fibrillar beta-amyloid and IgGs are differentially regulated by proinflammatory cytokines. J Neurosci 25(36):8240-9.

Koenigsknecht J, Landreth G. 2004. Microglial phagocytosis of fibrillar beta-amyloid through a beta1 integrin-dependent mechanism. J Neurosci 24(44):9838-46.

Koizumi S, Ohsawa K, Inoue K, Kohsaka S. 2013. Purinergic receptors in microglia: functional modal shifts of microglia mediated by P2 and P1 receptors. Glia 61(1):47-54.

Koizumi S, Shigemoto-Mogami Y, Nasu-Tada K, Shinozaki Y, Ohsawa K, Tsuda M, Joshi BV, Jacobson KA, Kohsaka S, Inoue K. 2007. UDP acting at P2Y6 receptors is a mediator of microglial phagocytosis. Nature 446(7139):1091-5.

Konig HG, Kogel D, Rami A, Prehn JH. 2005. TGF-{beta}1 activates two distinct type I receptors in neurons: implications for neuronal NF-{kappa}B signaling. J Cell Biol 168(7):1077-86.

Koning N, Swaab DF, Hoek RM, Huitinga I. 2009. Distribution of the immune inhibitory molecules CD200 and CD200R in the normal central nervous system and multiple sclerosis lesions suggests neuron-glia and glia-glia interactions. J Neuropathol Exp Neurol 68(2):159-67.

Korn T, Magnus T, Jung S. 2005. Autoantigen specific T cells inhibit glutamate uptake in astrocytes by decreasing expression of astrocytic glutamate transporter GLAST: a mechanism mediated by tumor necrosis factor-alpha. FASEB J 19(13):1878-80.

Kraft AD, Harry GJ. 2011. Features of microglia and neuroinflammation relevant to environmental exposure and neurotoxicity. Int J Environ Res Public Health 8(7):2980-3018.

Krause DL, Muller N. 2010. Neuroinflammation, microglia and implications for anti-inflammatory treatment in Alzheimer's disease. Int J Alzheimers Dis 2010.

Kreutz F, Frozza RL, Breier AC, de Oliveira VA, Horn AP, Pettenuzzo LF, Netto CA, Salbego CG, Trindade VM. 2011. Amyloid-beta induced toxicity involves ganglioside expression and is sensitive to GM1 neuroprotective action. Neurochem Int 59(5):648-55.

Kruger L, Mandelkow EM. 2015. Tau neurotoxicity and rescue in animal models of human Tauopathies. Curr Opin Neurobiol 36:52-58.

LaFerla FM, Green KN, Oddo S. 2007. Intracellular amyloid-beta in Alzheimer's disease. Nat Rev Neurosci 8(7):499-509.

Lagos-Quintana M, Rauhut R, Yalcin A, Meyer J, Lendeckel W, Tuschl T. 2002. Identification of tissue-specific microRNAs from mouse. Curr Biol 12(9):735-9.

Lambert MP, Barlow AK, Chromy BA, Edwards C, Freed R, Liosatos M, Morgan TE, Rozovsky I, Trommer B, Viola KL and others. 1998. Diffusible, nonfibrillar ligands derived from Abeta1-42 are potent central nervous system neurotoxins. Proc Natl Acad Sci U S A 95(11):6448-53.

Landreth GE, Reed-Geaghan EG. 2009. Toll-like receptors in Alzheimer's disease. Curr Top Microbiol Immunol 336:137-53.

Langa KM, Larson EB, Crimmins EM, Faul JD, Levine DA, Kabeto MU, Weir DR. 2016. A Comparison of the Prevalence of Dementia in the United States in 2000 and 2012. JAMA Intern Med 177(1):51-58.

Lasiene J, Matsui A, Sawa Y, Wong F, Horner PJ. 2009. Age-related myelin dynamics revealed by increased oligodendrogenesis and short internodes. Aging Cell 8(2):201-13.

Lawson LJ, Perry VH, Dri P, Gordon S. 1990. Heterogeneity in the distribution and morphology of microglia in the normal adult mouse brain. Neuroscience 39(1):151-70.

Lee CY, Landreth GE. 2010. The role of microglia in amyloid clearance from the AD brain. J Neural Transm (Vienna) 117(8):949-60.

Lee S, Varvel NH, Konerth ME, Xu G, Cardona AE, Ransohoff RM, Lamb BT. 2010. CX3CR1 deficiency alters microglial activation and reduces beta-amyloid deposition in two Alzheimer's disease mouse models. Am J Pathol 177(5):2549-62.

Lehnardt S, Massillon L, Follett P, Jensen FE, Ratan R, Rosenberg PA, Volpe JJ, Vartanian T. 2003. Activation of innate immunity in the CNS triggers

neurodegeneration through a Toll-like receptor 4-dependent pathway. Proc Natl Acad Sci U S A 100(14):8514-9.

Leonardi-Essmann F, Emig M, Kitamura Y, Spanagel R, Gebicke-Haerter PJ. 2005. Fractalkine-upregulated milk-fat globule EGF factor-8 protein in cultured rat microglia. J Neuroimmunol 160(1-2):92-101.

Lesne S, Koh MT, Kotilinek L, Kayed R, Glabe CG, Yang A, Gallagher M, Ashe KH. 2006. A specific amyloid-beta protein assembly in the brain impairs memory. Nature 440(7082):352-7.

Leuner K, Schutt T, Kurz C, Eckert SH, Schiller C, Occhipinti A, Mai S, Jendrach M, Eckert GP, Kruse SE and others. 2012. Mitochondrion-derived reactive oxygen species lead to enhanced amyloid beta formation. Antioxid Redox Signal 16(12):1421-33.

Lewczuk P, Esselmann H, Otto M, Maler JM, Henkel AW, Henkel MK, Eikenberg O, Antz C, Krause WR, Reulbach U and others. 2004. Neurochemical diagnosis of Alzheimer's dementia by CSF Abeta42, Abeta42/Abeta40 ratio and total tau. Neurobiol Aging 25(3):273-81.

Li YY, Cui JG, Dua P, Pogue AI, Bhattacharjee S, Lukiw WJ. 2011. Differential expression of miRNA-146a-regulated inflammatory genes in human primary neural, astroglial and microglial cells. Neurosci Lett 499(2):109-13.

Liu CC, Kanekiyo T, Xu H, Bu G. 2013. Apolipoprotein E and Alzheimer disease: risk, mechanisms and therapy. Nat Rev Neurol 9(2):106-18.

Liu G, Abraham E. 2013. MicroRNAs in immune response and macrophage polarization. Arterioscler Thromb Vasc Biol 33(2):170-7.

Liu GJ, Nagarajah R, Banati RB, Bennett MR. 2009. Glutamate induces directed chemotaxis of microglia. Eur J Neurosci 29(6):1108-18.

Liu J, Jin X, Liu KJ, Liu W. 2012a. Matrix metalloproteinase-2-mediated occludin degradation and caveolin-1-mediated claudin-5 redistribution contribute to blood-brain barrier damage in early ischemic stroke stage. J Neurosci 32(9):3044-57.

Liu S, Liu Y, Hao W, Wolf L, Kiliaan AJ, Penke B, Rube CE, Walter J, Heneka MT, Hartmann T and others. 2012b. TLR2 is a primary receptor for Alzheimer's amyloid beta peptide to trigger neuroinflammatory activation. J Immunol 188(3):1098-107.

Liu Y, Walter S, Stagi M, Cherny D, Letiembre M, Schulz-Schaeffer W, Heine H, Penke B, Neumann H, Fassbender K. 2005. LPS receptor (CD14): a receptor for phagocytosis of Alzheimer's amyloid peptide. Brain 128(Pt 8):1778-89.

Lopategui Cabezas I, Herrera Batista A, Pentón Rol G. 2012. The role of microglia cells in Alzheimer's disease: Potential therapeutic implications. Neurologia [Epub ahead of print].

Lotz M, Ebert S, Esselmann H, Iliev AI, Prinz M, Wiazewicz N, Wiltfang J, Gerber J, Nau R. 2005. Amyloid beta peptide 1-40 enhances the action of Toll-like receptor-2 and -4 agonists but antagonizes Toll-like receptor-9-induced inflammation in primary mouse microglial cell cultures. J Neurochem 94(2):289-98.

Lu C, Huang X, Zhang X, Roensch K, Cao Q, Nakayama KI, Blazar BR, Zeng Y, Zhou X. 2011. miR-221 and miR-155 regulate human dendritic cell development, apoptosis, and IL-12 production through targeting of p27kip1, KPC1, and SOCS-1. Blood 117(16):4293-303.

Lucas SM, Rothwell NJ, Gibson RM. 2006. The role of inflammation in CNS injury and disease. Br J Pharmacol 147 Suppl 1:S232-40.

Lucin KM, O'Brien CE, Bieri G, Czirr E, Mosher KI, Abbey RJ, Mastroeni DF, Rogers J, Spencer B, Masliah E and others. 2013. Microglial beclin 1 regulates retromer trafficking and phagocytosis and is impaired in Alzheimer's disease. Neuron 79(5):873-86.

Lue LF, Kuo YM, Beach T, Walker DG. 2010. Microglia activation and anti-inflammatory regulation in Alzheimer's disease. Mol Neurobiol 41(2-3):115-28.

Lue LF, Rydel R, Brigham EF, Yang LB, Hampel H, Murphy GM, Jr., Brachova L, Yan SD, Walker DG, Shen Y and others. 2001. Inflammatory repertoire of Alzheimer's disease and nondemented elderly microglia in vitro. Glia 35(1):72-9.

Lukiw WJ, Zhao Y, Cui JG. 2008. An NF-kappaB-sensitive micro RNA-146a-mediated inflammatory circuit in Alzheimer disease and in stressed human brain cells. J Biol Chem 283(46):31315-22.

Lynch AM, Murphy KJ, Deighan BF, O'Reilly JA, Gun'ko YK, Cowley TR, Gonzalez-Reyes RE, Lynch MA. 2010. The impact of glial activation in the aging brain. Aging Dis 1(3):262-78.

Lyons A, Downer EJ, Crotty S, Nolan YM, Mills KH, Lynch MA. 2007a. CD200 ligand receptor interaction modulates microglial activation in vivo and in vitro: a role for IL-4. J Neurosci 27(31):8309-13.

Lyons A, Griffin RJ, Costelloe CE, Clarke RM, Lynch MA. 2007b. IL-4 attenuates the neuroinflammation induced by amyloid-beta in vivo and in vitro. J Neurochem 101(3):771-81.

Lyons A, McQuillan K, Deighan BF, O'Reilly JA, Downer EJ, Murphy AC, Watson M, Piazza A, O'Connell F, Griffin R and others. 2009. Decreased neuronal CD200 expression in IL-4-deficient mice results in increased neuroinflammation in response to lipopolysaccharide. Brain Behav Immun 23(7):1020-7.

Macfarlane LA, Murphy PR. 2010. MicroRNA: Biogenesis, Function and Role in Cancer. Curr Genomics 11(7):537-61.

Maeda T, Inagaki M, Fujita Y, Kimoto T, Tanabe-Fujimura C, Zou K, Liu J, Liu S, Komano H. 2016. ATP increases the migration of microglia across the brain endothelial cell monolayer. Biosci Rep.

Maezawa I, Hong HS, Liu R, Wu CY, Cheng RH, Kung MP, Kung HF, Lam KS, Oddo S, Laferla FM and others. 2008. Congo red and thioflavin-T analogs detect Abeta oligomers. J Neurochem 104(2):457-68.

Maezawa I, Zimin PI, Wulff H, Jin LW. 2011. Amyloid-beta protein oligomer at low nanomolar concentrations activates microglia and induces microglial neurotoxicity. J Biol Chem 286(5):3693-706.

Majumdar A, Chung H, Dolios G, Wang R, Asamoah N, Lobel P, Maxfield FR. 2008. Degradation of fibrillar forms of Alzheimer's amyloid beta-peptide by macrophages. Neurobiol Aging 29(5):707-15.

Majumdar A, Cruz D, Asamoah N, Buxbaum A, Sohar I, Lobel P, Maxfield FR. 2007. Activation of microglia acidifies lysosomes and leads to degradation of Alzheimer amyloid fibrils. Mol Biol Cell 18(4):1490-6.

Manczak M, Anekonda TS, Henson E, Park BS, Quinn J, Reddy PH. 2006. Mitochondria are a direct site of A beta accumulation in Alzheimer's disease neurons: implications for free radical generation and oxidative damage in disease progression. Hum Mol Genet 15(9):1437-49.

Mandrekar S, Jiang Q, Lee CY, Koenigsknecht-Talboo J, Holtzman DM, Landreth GE. 2009. Microglia mediate the clearance of soluble Abeta through fluid phase macropinocytosis. J Neurosci 29(13):4252-62.

Manocha GD, Puig KL, Austin SA, Seyb K, Glicksman MA, Combs CK. 2015. Characterization of Novel Src Family Kinase Inhibitors to Attenuate Microgliosis. PLoS One 10(7):e0132604.

Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M. 2004. The chemokine system in diverse forms of macrophage activation and polarization. Trends Immunol 25(12):677-86.

Maruszak A, Zekanowski C. 2011. Mitochondrial dysfunction and Alzheimer's disease. Prog Neuropsychopharmacol Biol Psychiatry 35(2):320-30.

Mawuenyega KG, Sigurdson W, Ovod V, Munsell L, Kasten T, Morris JC, Yarasheski KE, Bateman RJ. 2010. Decreased clearance of CNS beta-amyloid in Alzheimer's disease. Science 330(6012):1774.

McArthur S, Cristante E, Paterno M, Christian H, Roncaroli F, Gillies GE, Solito E. 2010. Annexin A1: a central player in the anti-inflammatory and neuroprotective role of microglia. J Immunol 185(10):6317-28.

McGeer PL, Itagaki S, McGeer EG. 1988. Expression of the histocompatibility glycoprotein HLA-DR in neurological disease. Acta Neuropathol 76(6):550-7.

McGeer PL, Itagaki S, Tago H, McGeer EG. 1987. Reactive microglia in patients with senile dementia of the Alzheimer type are positive for the histocompatibility glycoprotein HLA-DR. Neurosci Lett 79(1-2):195-200.

McGeer PL, Rogers J, McGeer EG. 2006. Inflammation, anti-inflammatory agents and Alzheimer disease: the last 12 years. J Alzheimers Dis 9(3 Suppl):271-6.

McKhann GM, Knopman DS, Chertkow H, Hyman BT, Jack CR, Jr., Kawas CH, Klunk WE, Koroshetz WJ, Manly JJ, Mayeux R and others. 2011. The diagnosis of dementia due to Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. Alzheimers Dement 7(3):263-9.

McMullan SM, Phanavanh B, Li GG, Barger SW. 2012. Metabotropic glutamate receptors inhibit microglial glutamate release. ASN Neuro 4(5).

Meda L, Cassatella MA, Szendrei GI, Otvos L, Jr., Baron P, Villalba M, Ferrari D, Rossi F. 1995. Activation of microglial cells by beta-amyloid protein and interferon-gamma. Nature 374(6523):647-50.

Meraz-Rios MA, Toral-Rios D, Franco-Bocanegra D, Villeda-Hernandez J, Campos-Pena V. 2013. Inflammatory process in Alzheimer's Disease. Front Integr Neurosci 7:59.

Merlo S, Sortino MA. 2012. Estrogen activates matrix metalloproteinases-2 and -9 to increase beta amyloid degradation. Mol Cell Neurosci 49(4):423-9.

Mesulam MM. 2000. A plasticity-based theory of the pathogenesis of Alzheimer's disease. Ann N Y Acad Sci 924:42-52.

Meyer-Luehmann M, Spires-Jones TL, Prada C, Garcia-Alloza M, de Calignon A, Rozkalne A, Koenigsknecht-Talboo J, Holtzman DM, Bacskai BJ, Hyman BT. 2008. Rapid appearance and local toxicity of amyloid-beta plaques in a mouse model of Alzheimer's disease. Nature 451(7179):720-4.

Miguel-Alvarez M, Santos-Lozano A, Sanchis-Gomar F, Fiuza-Luces C, Pareja-Galeano H, Garatachea N, Lucia A. 2015. Non-steroidal anti-inflammatory drugs as a treatment for Alzheimer's disease: a systematic review and meta-analysis of treatment effect. Drugs Aging 32(2):139-47.

Miller KR, Streit WJ. 2007. The effects of aging, injury and disease on microglial function: a case for cellular senescence. Neuron Glia Biol 3(3):245-53.

Miners JS, Baig S, Palmer J, Palmer LE, Kehoe PG, Love S. 2008. Abeta-degrading enzymes in Alzheimer's disease. Brain Pathol 18(2):240-52.

Minghetti L, Ajmone-Cat MA, De Berardinis MA, De Simone R. 2005. Microglial activation in chronic neurodegenerative diseases: roles of apoptotic neurons and chronic stimulation. Brain Res Brain Res Rev 48(2):251-6.

Minghetti L, Pocchiari M. 2007. Cyclooxygenase-2, prostaglandin E2, and microglial activation in prion diseases. Int Rev Neurobiol 82:265-75.

Mizuno T. 2012. The biphasic role of microglia in Alzheimer's disease. Int J Alzheimers Dis 2012:737846.

Mohmmad Abdul H, Butterfield DA. 2005. Protection against amyloid beta-peptide (1-42)-induced loss of phospholipid asymmetry in synaptosomal membranes by tricyclodecan-9-xanthogenate (D609) and ferulic acid ethyl ester: implications for Alzheimer's disease. Biochim Biophys Acta 1741(1-2):140-8.

Montine TJ, Peskind ER, Quinn JF, Wilson AM, Montine KS, Galasko D. 2011. Increased cerebrospinal fluid F2-isoprostanes are associated with aging and latent Alzheimer's disease as identified by biomarkers. Neuromolecular Med 13(1):37-43.

Morgan D. 2009. The role of microglia in antibody-mediated clearance of amyloid-beta from the brain. CNS Neurol Disord Drug Targets 8(1):7-15.
Mori T, Koyama N, Arendash GW, Horikoshi-Sakuraba Y, Tan J, Town T. 2010. Overexpression of human S100B exacerbates cerebral amyloidosis and gliosis in the Tg2576 mouse model of Alzheimer's disease. Glia 58(3):300-14.
Morrissette DA, Parachikova A, Green KN, LaFerla FM. 2009. Relevance of transgenic mouse models to human Alzheimer disease. J Biol Chem 284(10):6033-7.
Muller S, Scaffidi P, Degryse B, Bonaldi T, Ronfani L, Agresti A, Beltrame M, Bianchi ME. 2001. New EMBO members' review: the double life of HMGB1 chromatin protein: architectural factor and extracellular signal. EMBO J 20(16):4337-40.
Murphy S. 2000. Production of nitric oxide by glial cells: regulation and potential roles in the CNS. Glia 29(1):1-13.
Nagase H, Visse R, Murphy G. 2006. Structure and function of matrix metalloproteinases and TIMPs. Cardiovasc Res 69(3):562-73.
Najem D, Bamji-Mirza M, Chang N, Liu QY, Zhang W. 2014. Insulin resistance, neuroinflammation, and Alzheimer's disease. Rev Neurosci 25(4):509-25.
Nakanishi H. 2003. Microglial functions and proteases. Mol Neurobiol 27(2):163-76.
Napoli I, Neumann H. 2009. Microglial clearance function in health and disease. Neuroscience 158(3):1030-8.
Nathalie P, Jean-Noel O. 2008. Processing of amyloid precursor protein and amyloid peptide neurotoxicity. Curr Alzheimer Res 5(2):92-9.
Nayak D, Huo Y, Kwang WX, Pushparaj PN, Kumar SD, Ling EA, Dheen ST. 2010. Sphingosine kinase 1 regulates the expression of proinflammatory cytokines and nitric oxide in activated microglia. Neuroscience 166(1):132-44.
Neher JJ, Neniskyte U, Brown GC. 2012. Primary phagocytosis of neurons by inflamed microglia: potential roles in neurodegeneration. Front Pharmacol 3:27.
Nelson L, Tabet N. 2015. Slowing the progression of Alzheimer's disease; what works? Ageing Res Rev 23(Pt B):193-209.
Neniskyte U, Neher JJ, Brown GC. 2011. Neuronal death induced by nanomolar amyloid beta is mediated by primary phagocytosis of neurons by microglia. J Biol Chem 286(46):39904-13.
Neumann H, Kotter MR, Franklin RJ. 2009. Debris clearance by microglia: an essential link between degeneration and regeneration. Brain 132(Pt 2):288-95.
Ni M, Aschner M. 2010. Neonatal rat primary microglia: isolation, culturing, and selected applications. Curr Protoc Toxicol Chapter 12:Unit 12 17.
Nicholson AM, Ferreira A. 2009. Increased membrane cholesterol might render mature hippocampal neurons more susceptible to beta-amyloid-induced calpain activation and tau toxicity. J Neurosci 29(14):4640-51.
Nimmerjahn A, Kirchhoff F, Helmchen F. 2005. Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. Science 308(5726):1314-8.
Njie EG, Boelen E, Stassen FR, Steinbusch HW, Borchelt DR, Streit WJ. 2012. Ex vivo cultures of microglia from young and aged rodent brain reveal age-related changes in microglial function. Neurobiol Aging 33(1):195 e1-12.
Noda M, Doi Y, Liang J, Kawanokuchi J, Sonobe Y, Takeuchi H, Mizuno T, Suzumura A. 2011. Fractalkine attenuates excito-neurotoxicity via microglial clearance of damaged neurons and antioxidant enzyme heme oxygenase-1 expression. J Biol Chem 286(3):2308-19.
Noda M, Suzumura A. 2012. Sweepers in the CNS: Microglial Migration and Phagocytosis in the Alzheimer Disease Pathogenesis. Int J Alzheimers Dis 2012:891087.
O'Brien RJ, Wong PC. 2011. Amyloid precursor protein processing and Alzheimer's disease. Annu Rev Neurosci 34:185-204.
O'Connell RM, Rao DS, Chaudhuri AA, Baltimore D. 2010. Physiological and pathological roles for microRNAs in the immune system. Nat Rev Immunol 10(2):111-22.

Oakley H, Cole SL, Logan S, Maus E, Shao P, Craft J, Guillozet-Bongaarts A, Ohno M, Disterhoft J, Van Eldik L and others. 2006. Intraneuronal beta-amyloid aggregates, neurodegeneration, and neuron loss in transgenic mice with five familial Alzheimer's disease mutations: potential factors in amyloid plaque formation. J Neurosci 26(40):10129-40.

Octave JN. 2005. [Alzheimer disease: cellular and molecular aspects]. Bull Mem Acad R Med Belg 160(10-12):445-9; discussion 450-1.

Oddo S, Caccamo A, Kitazawa M, Tseng BP, LaFerla FM. 2003a. Amyloid deposition precedes tangle formation in a triple transgenic model of Alzheimer's disease. Neurobiol Aging 24(8):1063-70.

Oddo S, Caccamo A, Shepherd JD, Murphy MP, Golde TE, Kayed R, Metherate R, Mattson MP, Akbari Y, LaFerla FM. 2003b. Triple-transgenic model of Alzheimer's disease with plaques and tangles: intracellular Abeta and synaptic dysfunction. Neuron 39(3):409-21.

Olah M, Biber K, Vinet J, Boddeke HW. 2011. Microglia phenotype diversity. CNS Neurol Disord Drug Targets 10(1):108-18.

Olson JK, Miller SD. 2004. Microglia initiate central nervous system innate and adaptive immune responses through multiple TLRs. J Immunol 173(6):3916-24.

Onyango IG, Khan SM. 2006. Oxidative stress, mitochondrial dysfunction, and stress signaling in Alzheimer's disease. Curr Alzheimer Res 3(4):339-49.

Osborn LM, Kamphuis W, Wadman WJ, Hol EM. 2016. Astrogliosis: An integral player in the pathogenesis of Alzheimer's disease. Prog Neurobiol.

Pan XD, Zhu YG, Lin N, Zhang J, Ye QY, Huang HP, Chen XC. 2011. Microglial phagocytosis induced by fibrillar beta-amyloid is attenuated by oligomeric beta-amyloid: implications for Alzheimer's disease. Mol Neurodegener 6:45.

Parachikova A, Agadjanyan MG, Cribbs DH, Blurton-Jones M, Perreau V, Rogers J, Beach TG, Cotman CW. 2007. Inflammatory changes parallel the early stages of Alzheimer disease. Neurobiol Aging 28(12):1821-33.

Park JS, Gamboni-Robertson F, He Q, Svetkauskaite D, Kim JY, Strassheim D, Sohn JW, Yamada S, Maruyama I, Banerjee A and others. 2006. High mobility group box 1 protein interacts with multiple Toll-like receptors. Am J Physiol Cell Physiol 290(3):C917-24.

Park JS, Svetkauskaite D, He Q, Kim JY, Strassheim D, Ishizaka A, Abraham E. 2004. Involvement of toll-like receptors 2 and 4 in cellular activation by high mobility group box 1 protein. J Biol Chem 279(9):7370-7.

Park S, Kim HT, Yun S, Kim IS, Lee J, Lee IS, Park KI. 2009. Growth factor-expressing human neural progenitor cell grafts protect motor neurons but do not ameliorate motor performance and survival in ALS mice. Exp Mol Med 41(7):487-500.

Parkhurst CN, Gan WB. 2010. Microglia dynamics and function in the CNS. Curr Opin Neurobiol 20(5):595-600.

Parkhurst CN, Yang G, Ninan I, Savas JN, Yates JR, 3rd, Lafaille JJ, Hempstead BL, Littman DR, Gan WB. 2013. Microglia promote learning-dependent synapse formation through brain-derived neurotrophic factor. Cell 155(7):1596-609.

Parsons CG, Danysz W, Dekundy A, Pulte I. 2013. Memantine and cholinesterase inhibitors: complementary mechanisms in the treatment of Alzheimer's disease. Neurotox Res 24(3):358-69.

Parvathy S, Rajadas J, Ryan H, Vaziri S, Anderson L, Murphy GM, Jr. 2009. Abeta peptide conformation determines uptake and interleukin-1alpha expression by primary microglial cells. Neurobiol Aging 30(11):1792-804.

Pasqualetti G, Brooks DJ, Edison P. 2015. The role of neuroinflammation in dementias. Curr Neurol Neurosci Rep 15(4):17.

Patel NS, Paris D, Mathura V, Quadros AN, Crawford FC, Mullan MJ. 2005. Inflammatory cytokine levels correlate with amyloid load in transgenic mouse models of Alzheimer's disease. J Neuroinflammation 2(1):9.

Peixoto CA, Nunes AK, Garcia-Osta A. 2015. Phosphodiesterase-5 Inhibitors: Action on the Signaling Pathways of Neuroinflammation, Neurodegeneration, and Cognition. Mediators Inflamm 2015:940207.

Perez-Gonzalez R, Gauthier SA, Kumar A, Levy E. 2012. The exosome secretory pathway transports amyloid precursor protein carboxyl-terminal fragments from the cell into the brain extracellular space. J Biol Chem 287(51):43108-15.

Perry VH, Cunningham C, Holmes C. 2007. Systemic infections and inflammation affect chronic neurodegeneration. Nat Rev Immunol 7(2):161-7.

Perry VH, Teeling J. 2013. Microglia and macrophages of the central nervous system: the contribution of microglia priming and systemic inflammation to chronic neurodegeneration. Semin Immunopathol 35(5):601-12.

Pike KE, Savage G, Villemagne VL, Ng S, Moss SA, Maruff P, Mathis CA, Klunk WE, Masters CL, Rowe CC. 2007. Beta-amyloid imaging and memory in non-demented individuals: evidence for preclinical Alzheimer's disease. Brain 130(Pt 11):2837-44.

Ponomarev ED, Veremeyko T, Barteneva N, Krichevsky AM, Weiner HL. 2011. MicroRNA-124 promotes microglia quiescence and suppresses EAE by deactivating macrophages via the C/EBP-alpha-PU.1 pathway. Nat Med 17(1):64-70.

Ponomarev ED, Veremeyko T, Weiner HL. 2013. MicroRNAs are universal regulators of differentiation, activation, and polarization of microglia and macrophages in normal and diseased CNS. Glia 61(1):91-103.

Puntener U, Booth SG, Perry VH, Teeling JL. 2012. Long-term impact of systemic bacterial infection on the cerebral vasculature and microglia. J Neuroinflammation 9:146.

Puzzo D, Arancio O. 2013. Amyloid-beta peptide: Dr. Jekyll or Mr. Hyde? J Alzheimers Dis 33 Suppl 1:S111-20.

Raes G, Noel W, Beschin A, Brys L, de Baetselier P, Hassanzadeh GH. 2002. FIZZ1 and Ym as tools to discriminate between differentially activated macrophages. Dev Immunol 9(3):151-9.

Raina P, Santaguida P, Ismaila A, Patterson C, Cowan D, Levine M, Booker L, Oremus M. 2008. Effectiveness of cholinesterase inhibitors and memantine for treating dementia: evidence review for a clinical practice guideline. Ann Intern Med 148(5):379-97.

Ralay Ranaivo H, Craft JM, Hu W, Guo L, Wing LK, Van Eldik LJ, Watterson DM. 2006. Glia as a therapeutic target: selective suppression of human amyloid-beta-induced upregulation of brain proinflammatory cytokine production attenuates neurodegeneration. J Neurosci 26(2):662-70.

Ralevic V, Burnstock G. 1998. Receptors for purines and pyrimidines. Pharmacol Rev 50(3):413-92.

Ransohoff RM, Perry VH. 2009. Microglial physiology: unique stimuli, specialized responses. Annu Rev Immunol 27:119-45.

Reddy PH, Beal MF. 2008. Amyloid beta, mitochondrial dysfunction and synaptic damage: implications for cognitive decline in aging and Alzheimer's disease. Trends Mol Med 14(2):45-53.

Reed-Geaghan EG, Savage JC, Hise AG, Landreth GE. 2009. CD14 and toll-like receptors 2 and 4 are required for fibrillar A{beta}-stimulated microglial activation. J Neurosci 29(38):11982-92.

Righi M, Mori L, De Libero G, Sironi M, Biondi A, Mantovani A, Donini SD, Ricciardi-Castagnoli P. 1989. Monokine production by microglial cell clones. Eur J Immunol 19(8):1443-8.

Rodriguez JJ, Witton J, Olabarria M, Noristani HN, Verkhratsky A. 2010. Increase in the density of resting microglia precedes neuritic plaque formation and microglial activation in a transgenic model of Alzheimer's disease. Cell Death Dis 1:e1.

Rogers JT, Morganti JM, Bachstetter AD, Hudson CE, Peters MM, Grimmig BA, Weeber EJ, Bickford PC, Gemma C. 2011. CX3CR1 deficiency leads to impairment of hippocampal cognitive function and synaptic plasticity. J Neurosci 31(45):16241-50.

Roher AE, Kasunic TC, Woods AS, Cotter RJ, Ball MJ, Fridman R. 1994. Proteolysis of A beta peptide from Alzheimer disease brain by gelatinase A. Biochem Biophys Res Commun 205(3):1755-61.

Roher AE, Weiss N, Kokjohn TA, Kuo YM, Kalback W, Anthony J, Watson D, Luehrs DC, Sue L, Walker D and others. 2002. Increased A beta peptides and reduced cholesterol and myelin proteins characterize white matter degeneration in Alzheimer's disease. Biochemistry 41(37):11080-90.

Rojanathammanee L, Floden AM, Manocha GD, Combs CK. 2015. Attenuation of microglial activation in a mouse model of Alzheimer's disease via NFAT inhibition. J Neuroinflammation 12:42.

Rosenmann H, Grigoriadis N, Karussis D, Boimel M, Touloumi O, Ovadia H, Abramsky O. 2006. Tauopathy-like abnormalities and neurologic deficits in mice immunized with neuronal tau protein. Arch Neurol 63(10):1459-67.

Roth AD, Ramirez G, Alarcon R, Von Bernhardi R. 2005. Oligodendrocytes damage in Alzheimer's disease: beta amyloid toxicity and inflammation. Biol Res 38(4):381-7.

Roy A, Fung YK, Liu X, Pahan K. 2006. Up-regulation of microglial CD11b expression by nitric oxide. J Biol Chem 281(21):14971-80.

Rubio-Perez JM, Morillas-Ruiz JM. 2012. A review: inflammatory process in Alzheimer's disease, role of cytokines. ScientificWorldJournal 2012:756357.

Ryu JK, Cho T, Choi HB, Jantaratnotai N, McLarnon JG. 2015. Pharmacological antagonism of interleukin-8 receptor CXCR2 inhibits inflammatory reactivity and is neuroprotective in an animal model of Alzheimer's disease. J Neuroinflammation 12:144.

Sabogal-Guaqueta AM, Osorio E, Cardona-Gomez GP. 2016. Linalool reverses neuropathological and behavioral impairments in old triple transgenic Alzheimer's mice. Neuropharmacology 102:111-20.

Salawu FK, Umar JT, Olokoba AB. 2011. Alzheimer's disease: a review of recent developments. Ann Afr Med 10(2):73-9.

Salminen A, Ojala J, Kauppinen A, Kaarniranta K, Suuronen T. 2009. Inflammation in Alzheimer's disease: amyloid-beta oligomers trigger innate immunity defence via pattern recognition receptors. Prog Neurobiol 87(3):181-94.

Samuels SE, Lipitz JB, Dahl G, Muller KJ. 2010. Neuroglial ATP release through innexin channels controls microglial cell movement to a nerve injury. J Gen Physiol 136(4):425-42.

Sanz JM, Chiozzi P, Ferrari D, Colaianna M, Idzko M, Falzoni S, Fellin R, Trabace L, Di Virgilio F. 2009. Activation of microglia by amyloid {beta} requires P2X7 receptor expression. J Immunol 182(7):4378-85.

Savonenko A, Xu GM, Melnikova T, Morton JL, Gonzales V, Wong MP, Price DL, Tang F, Markowska AL, Borchelt DR. 2005. Episodic-like memory deficits in the APPswe/PS1dE9 mouse model of Alzheimer's disease: relationships to beta-amyloid deposition and neurotransmitter abnormalities. Neurobiol Dis 18(3):602-17.

Schafer DP, Lehrman EK, Kautzman AG, Koyama R, Mardinly AR, Yamasaki R, Ransohoff RM, Greenberg ME, Barres BA, Stevens B. 2012. Microglia sculpt postnatal neural circuits in an activity and complement-dependent manner. Neuron 74(4):691-705.

Schneider EL, Mitsui Y. 1976. The relationship between in vitro cellular aging and in vivo human age. Proc Natl Acad Sci U S A 73(10):3584-8.

Schrag M, Sharma S, Brown-Borg H, Ghribi O. 2008. Hippocampus of Ames dwarf mice is resistant to beta-amyloid-induced tau hyperphosphorylation and changes in apoptosis-regulatory protein levels. Hippocampus 18(3):239-44.

Schuitemaker A, van der Doef TF, Boellaard R, van der Flier WM, Yaqub M, Windhorst AD, Barkhof F, Jonker C, Kloet RW, Lammertsma AA and others. 2012. Microglial activation in healthy aging. Neurobiol Aging 33(6):1067-72.

Schwab C, Klegeris A, McGeer PL. 2009. Inflammation in transgenic mouse models of neurodegenerative disorders. Biochim Biophys Acta 1802(10):889-902.

Schwartz M, Butovsky O, Bruck W, Hanisch UK. 2006. Microglial phenotype: is the commitment reversible? Trends Neurosci 29(2):68-74.

Selkoe DJ. 2001. Alzheimer's disease: genes, proteins, and therapy. Physiol Rev 81(2):741-66.

Sha S, Xing XN, Cao YP. 2014. Active immunotherapy facilitates Abeta plaque removal following through microglial activation without obvious T cells infiltrating the CNS. J Neuroimmunol 274(1-2):62-70.

Shankar GM, Li S, Mehta TH, Garcia-Munoz A, Shepardson NE, Smith I, Brett FM, Farrell MA, Rowan MJ, Lemere CA and others. 2008. Amyloid-beta protein dimers isolated directly from Alzheimer's brains impair synaptic plasticity and memory. Nat Med 14(8):837-42.

Sheng M, Sabatini BL, Sudhof TC. 2012. Synapses and Alzheimer's disease. Cold Spring Harb Perspect Biol 4(5).

Siciliano R, Barone E, Calabrese V, Rispoli V, Butterfield DA, Mancuso C. 2011. Experimental research on nitric oxide and the therapy of Alzheimer disease: a challenging bridge. CNS Neurol Disord Drug Targets 10(7):766-76.

Silva DF, Selfridge JE, Lu J, E L, Cardoso SM, Swerdlow RH. 2012. Mitochondrial abnormalities in Alzheimer's disease: possible targets for therapeutic intervention. Adv Pharmacol 64:83-126.

Simic G, Babic Leko M, Wray S, Harrington C, Delalle I, Jovanov-Milosevic N, Bazadona D, Buee L, de Silva R, Di Giovanni G and others. 2016. Tau Protein Hyperphosphorylation and Aggregation in Alzheimer's Disease and Other Tauopathies, and Possible Neuroprotective Strategies. Biomolecules 6(1).

Skok M, Lykhmus O. 2016. The Role Of alpha7 Nicotinic Acetylcholine Receptors And alpha7-Specific Antibodies In Neuroinflammation Related To Alzheimer Disease. Curr Pharm Des.

Sokolowski JD, Mandell JW. 2011. Phagocytic clearance in neurodegeneration. Am J Pathol 178(4):1416-28.

Solito E, Sastre M. 2012. Microglia function in Alzheimer's disease. Front Pharmacol 3:14.

Sondag CM, Dhawan G, Combs CK. 2009. Beta amyloid oligomers and fibrils stimulate differential activation of primary microglia. J Neuroinflammation 6:1.

Southam KA, Vincent AJ, Small DH. 2016. Do Microglia Default on Network Maintenance in Alzheimer's Disease? J Alzheimers Dis 51(3):657-69.

Sperlagh B, Illes P. 2007. Purinergic modulation of microglial cell activation. Purinergic Signal 3(1-2):117-27.

Sperling RA, Aisen PS, Beckett LA, Bennett DA, Craft S, Fagan AM, Iwatsubo T, Jack CR, Jr., Kaye J, Montine TJ and others. 2011. Toward defining the preclinical stages of Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. Alzheimers Dement 7(3):280-92.

Spuch C, Ortolano S, Navarro C. 2012. New insights in the amyloid-Beta interaction with mitochondria. J Aging Res 2012:324968.

Staal JA, Alexander SR, Liu Y, Dickson TD, Vickers JC. 2011. Characterization of cortical neuronal and glial alterations during culture of organotypic whole brain slices from neonatal and mature mice. PLoS One 6(7):e22040.

Stansley B, Post J, Hensley K. 2012. A comparative review of cell culture systems for the study of microglial biology in Alzheimer's disease. J Neuroinflammation 9:115.

Sterniczuk R, Antle MC, Laferla FM, Dyck RH. 2010a. Characterization of the 3xTg-AD mouse model of Alzheimer's disease: part 2. Behavioral and cognitive changes. Brain Res 1348:149-55.

Sterniczuk R, Dyck RH, Laferla FM, Antle MC. 2010b. Characterization of the 3xTg-AD mouse model of Alzheimer's disease: part 1. Circadian changes. Brain Res 1348:139-48.

Stewart CR, Stuart LM, Wilkinson K, van Gils JM, Deng J, Halle A, Rayner KJ, Boyer L, Zhong R, Frazier WA and others. 2010. CD36 ligands promote sterile inflammation through assembly of a Toll-like receptor 4 and 6 heterodimer. Nat Immunol 11(2):155-61.

Stone J. 2008. What initiates the formation of senile plaques? The origin of Alzheimer-like dementias in capillary haemorrhages. Med Hypotheses 71(3):347-59.

Stone J, Johnstone DM, Mitrofanis J, O'Rourke M. 2015. The mechanical cause of age-related dementia (Alzheimer's disease): the brain is destroyed by the pulse. J Alzheimers Dis 44(2):355-73.

Street JM, Barran PE, Mackay CL, Weidt S, Balmforth C, Walsh TS, Chalmers RT, Webb DJ, Dear JW. 2012. Identification and proteomic profiling of exosomes in human cerebrospinal fluid. J Transl Med 10:5.

Streit WJ. 2002. Microglia as neuroprotective, immunocompetent cells of the CNS. Glia 40(2):133-9.

Streit WJ. 2004. Microglia and Alzheimer's disease pathogenesis. J Neurosci Res 77(1):1-8.

Streit WJ. 2005. Microglia and neuroprotection: implications for Alzheimer's disease. Brain Res Brain Res Rev 48(2):234-9.

Streit WJ. 2006. Microglial senescence: does the brain's immune system have an expiration date? Trends Neurosci 29(9):506-10.

Streit WJ, Braak H, Xue QS, Bechmann I. 2009. Dystrophic (senescent) rather than activated microglial cells are associated with tau pathology and likely precede neurodegeneration in Alzheimer's disease. Acta Neuropathol 118(4):475-85.

Streit WJ, Sammons NW, Kuhns AJ, Sparks DL. 2004. Dystrophic microglia in the aging human brain. Glia 45(2):208-12.

Streit WJ, Walter SA, Pennell NA. 1999. Reactive microgliosis. Prog Neurobiol 57(6):563-81.

Streit WJ, Xue QS. 2009. Life and death of microglia. J Neuroimmune Pharmacol 4(4):371-9.

Streit WJ, Xue QS. 2012. Alzheimer's disease, neuroprotection, and CNS immunosenescence. Front Pharmacol 3:138.

Streit WJ, Xue QS, Tischer J, Bechmann I. 2014. Microglial pathology. Acta Neuropathol Commun 2:142.

Su T, Paradiso B, Long YS, Liao WP, Simonato M. 2011. Evaluation of cell damage in organotypic hippocampal slice culture from adult mouse: a potential model system to study neuroprotection. Brain Res 1385:68-76.

Su W, Aloi MS, Garden GA. 2015. MicroRNAs mediating CNS inflammation: Small regulators with powerful potential. Brain Behav Immun.

Sudduth TL, Schmitt FA, Nelson PT, Wilcock DM. 2013. Neuroinflammatory phenotype in early Alzheimer's disease. Neurobiol Aging 34(4):1051-9.

Szczepanik AM, Funes S, Petko W, Ringheim GE. 2001. IL-4, IL-10 and IL-13 modulate A beta(1--42)-induced cytokine and chemokine production in primary murine microglia and a human monocyte cell line. J Neuroimmunol 113(1):49-62.

Tahara K, Kim HD, Jin JJ, Maxwell JA, Li L, Fukuchi K. 2006. Role of toll-like receptor signalling in Abeta uptake and clearance. Brain 129(Pt 11):3006-19.

Takata K, Kitamura Y, Kakimura J, Shibagaki K, Tsuchiya D, Taniguchi T, Smith MA, Perry G, Shimohama S. 2003a. Role of high mobility group protein-1 (HMG1) in amyloid-beta homeostasis. Biochem Biophys Res Commun 301(3):699-703.

Takata K, Kitamura Y, Umeki M, Tsuchiya D, Kakimura J, Taniguchi T, Gebicke-Haerter PJ, Shimohama S. 2003b. Possible involvement of small oligomers of amyloid-beta peptides in 15-deoxy-delta 12,14 prostaglandin J2-sensitive microglial activation. J Pharmacol Sci 91(4):330-3.

Takata K, Takada T, Ito A, Asai M, Tawa M, Saito Y, Ashihara E, Tomimoto H, Kitamura Y, Shimohama S. 2012. Microglial Amyloid-beta1-40 Phagocytosis Dysfunction Is Caused by High-Mobility Group Box Protein-1: Implications for the Pathological Progression of Alzheimer's Disease. Int J Alzheimers Dis 2012:685739.

Takeuchi H, Jin S, Wang J, Zhang G, Kawanokuchi J, Kuno R, Sonobe Y, Mizuno T, Suzumura A. 2006. Tumor necrosis factor-alpha induces neurotoxicity via glutamate release from hemichannels of activated microglia in an autocrine manner. J Biol Chem 281(30):21362-8.

Tambuyzer BR, Ponsaerts P, Nouwen EJ. 2009. Microglia: gatekeepers of central nervous system immunology. J Leukoc Biol 85(3):352-70.

Tan ZS, Beiser AS, Vasan RS, Roubenoff R, Dinarello CA, Harris TB, Benjamin EJ, Au R, Kiel DP, Wolf PA and others. 2007. Inflammatory markers and the risk of Alzheimer disease: the Framingham Study. Neurology 68(22):1902-8.

Tomic JL, Pensalfini A, Head E, Glabe CG. 2009. Soluble fibrillar oligomer levels are elevated in Alzheimer's disease brain and correlate with cognitive dysfunction. Neurobiol Dis 35(3):352-8.

Tremblay ME, Lowery RL, Majewska AK. 2010. Microglial interactions with synapses are modulated by visual experience. PLoS Biol 8(11):e1000527.

Troquier L, Caillierez R, Burnouf S, Fernandez-Gomez FJ, Grosjean ME, Zommer N, Sergeant N, Schraen-Maschke S, Blum D, Buee L. 2012. Targeting phospho-Ser422 by active Tau Immunotherapy in the THYTau22 mouse model: a suitable therapeutic approach. Curr Alzheimer Res 9(4):397-405.

Trotti D, Danbolt NC, Volterra A. 1998. Glutamate transporters are oxidant-vulnerable: a molecular link between oxidative and excitotoxic neurodegeneration? Trends Pharmacol Sci 19(8):328-34.

van Landeghem FK, Stover JF, Bechmann I, Bruck W, Unterberg A, Buhrer C, von Deimling A. 2001. Early expression of glutamate transporter proteins in ramified microglia after controlled cortical impact injury in the rat. Glia 35(3):167-79.

Vandenberghe R, Tournoy J. 2005. Cognitive aging and Alzheimer's disease. Postgrad Med J 81(956):343-52.

Varnum MM, Ikezu T. 2012. The classification of microglial activation phenotypes on neurodegeneration and regeneration in Alzheimer's disease brain. Arch Immunol Ther Exp (Warsz) 60(4):251-66.

Varvel NH, Bhaskar K, Kounnas MZ, Wagner SL, Yang Y, Lamb BT, Herrup K. 2009. NSAIDs prevent, but do not reverse, neuronal cell cycle reentry in a mouse model of Alzheimer disease. J Clin Invest 119(12):3692-702.

Vassar R, Bennett BD, Babu-Khan S, Kahn S, Mendiaz EA, Denis P, Teplow DB, Ross S, Amarante P, Loeloff R and others. 1999. Beta-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE. Science 286(5440):735-41.

Vella LJ, Greenwood DL, Cappai R, Scheerlinck JP, Hill AF. 2008. Enrichment of prion protein in exosomes derived from ovine cerebral spinal fluid. Vet Immunol Immunopathol 124(3-4):385-93.

Vella LJ, Hill AF, Cheng L. 2016. Focus on Extracellular Vesicles: Exosomes and Their Role in Protein Trafficking and Biomarker Potential in Alzheimer's and Parkinson's Disease. Int J Mol Sci 17(2).

Vilhardt F. 2005. Microglia: phagocyte and glia cell. Int J Biochem Cell Biol 37(1):17-21.

Villoslada P, Moreno B, Melero I, Pablos JL, Martino G, Uccelli A, Montalban X, Avila J, Rivest S, Acarin L and others. 2008. Immunotherapy for neurological diseases. Clin Immunol 128(3):294-305.

Vincent C, Siddiqui TA, Schlichter LC. 2012. Podosomes in migrating microglia: components and matrix degradation. J Neuroinflammation 9:190.

Volianskis A, Kostner R, Molgaard M, Hass S, Jensen MS. 2010. Episodic memory deficits are not related to altered glutamatergic synaptic transmission and plasticity in the CA1 hippocampus of the APPswe/PS1deltaE9-deleted transgenic mice model of ss-amyloidosis. Neurobiol Aging 31(7):1173-87.

von Bernhardi R. 2010. Immunotherapy in Alzheimer's disease: where do we stand? Where should we go? J Alzheimers Dis 19(2):405-21.

von Bernhardi R, Cornejo F, Parada GE, Eugenin J. 2015. Role of TGFbeta signaling in the pathogenesis of Alzheimer's disease. Front Cell Neurosci 9:426.

von Bernhardi R, Tichauer J, Eugenin-von Bernhardi L. 2011. Proliferating culture of aged microglia for the study of neurodegenerative diseases. J Neurosci Methods 202(1):65-9.

Vos SJ, Verhey F, Frolich L, Kornhuber J, Wiltfang J, Maier W, Peters O, Ruther E, Nobili F, Morbelli S and others. 2015. Prevalence and prognosis of Alzheimer's disease at the mild cognitive impairment stage. Brain 138(Pt 5):1327-38.

Walker DG, Dalsing-Hernandez JE, Campbell NA, Lue LF. 2009. Decreased expression of CD200 and CD200 receptor in Alzheimer's disease: a potential mechanism leading to chronic inflammation. Exp Neurol 215(1):5-19.

Walsh DM, Hartley DM, Kusumoto Y, Fezoui Y, Condron MM, Lomakin A, Benedek GB, Selkoe DJ, Teplow DB. 1999. Amyloid beta-protein fibrillogenesis. Structure and biological activity of protofibrillar intermediates. J Biol Chem 274(36):25945-52.

Walsh DM, Selkoe DJ. 2007. A beta oligomers - a decade of discovery. J Neurochem 101(5):1172-84.

Walsh DM, Tseng BP, Rydel RE, Podlisny MB, Selkoe DJ. 2000. The oligomerization of amyloid beta-protein begins intracellularly in cells derived from human brain. Biochemistry 39(35):10831-9.

Walter S, Letiembre M, Liu Y, Heine H, Penke B, Hao W, Bode B, Manietta N, Walter J, Schulz-Schuffer W and others. 2007. Role of the toll-like receptor 4 in neuroinflammation in Alzheimer's disease. Cell Physiol Biochem 20(6):947-56.

Wang M, Takagi G, Asai K, Resuello RG, Natividad FF, Vatner DE, Vatner SF, Lakatta EG. 2003. Aging increases aortic MMP-2 activity and angiotensin II in nonhuman primates. Hypertension 41(6):1308-16.

Wang XX, Tan MS, Yu JT, Tan L. 2014. Matrix metalloproteinases and their multiple roles in Alzheimer's disease. Biomed Res Int 2014:908636.

Weekman EM, Sudduth TL, Abner EL, Popa GJ, Mendenhall MD, Brothers HM, Braun K, Greenstein A, Wilcock DM. 2014. Transition from an M1 to a mixed neuroinflammatory phenotype increases amyloid deposition in APP/PS1 transgenic mice. J Neuroinflammation 11:127.

Weggen S, Beher D. 2012. Molecular consequences of amyloid precursor protein and presenilin mutations causing autosomal-dominant Alzheimer's disease. Alzheimers Res Ther 4(2):9.

Wenk GL. 2006. Neuropathologic changes in Alzheimer's disease: potential targets for treatment. J Clin Psychiatry 67 Suppl 3:3-7; quiz 23.

Wes PD, Holtman IR, Boddeke EW, Moller T, Eggen BJ. 2016. Next generation transcriptomics and genomics elucidate biological complexity of microglia in health and disease. Glia 64(2):197-213.

Wesson DW, Nixon RA, Levy E, Wilson DA. 2011. Mechanisms of neural and behavioral dysfunction in Alzheimer's disease. Mol Neurobiol 43(3):163-79.

Westerman MA, Cooper-Blacketer D, Mariash A, Kotilinek L, Kawarabayashi T, Younkin LH, Carlson GA, Younkin SG, Ashe KH. 2002. The relationship between Abeta

and memory in the Tg2576 mouse model of Alzheimer's disease. J Neurosci 22(5):1858-67.

Wilhelmi E, Schoder UH, Benabdallah A, Sieg F, Breder J, Reymann KG. 2002. Organotypic brain-slice cultures from adult rats: approaches for a prolonged culture time. Altern Lab Anim 30(3):275-83.

Wilkinson K, El Khoury J. 2012. Microglial scavenger receptors and their roles in the pathogenesis of Alzheimer's disease. Int J Alzheimers Dis 2012:489456.

Wilson EN, Abela AR, Do Carmo S, Allard S, Marks AR, Welikovitch LA, Ducatenzeiler A, Chudasama Y, Cuello AC. 2016. Intraneuronal Amyloid Beta Accumulation Disrupts Hippocampal CRTC1-Dependent Gene Expression and Cognitive Function in a Rat Model of Alzheimer Disease. Cereb Cortex.

Witting W, Kwa IH, Eikelenboom P, Mirmiran M, Swaab DF. 1990. Alterations in the circadian rest-activity rhythm in aging and Alzheimer's disease. Biol Psychiatry 27(6):563-72.

Wojtera M, Sobow T, Kloszewska I, Liberski PP, Brown DR, Sikorska B. 2012. Expression of immunohistochemical markers on microglia in Creutzfeldt-Jakob disease and Alzheimer's disease: morphometric study and review of the literature. Folia Neuropathol 50(1):74-84.

Wong WT. 2013. Microglial aging in the healthy CNS: phenotypes, drivers, and rejuvenation. Front Cell Neurosci 7:22.

Wu YY, Hsu JL, Wang HC, Wu SJ, Hong CJ, Cheng IH. 2015. Alterations of the Neuroinflammatory Markers IL-6 and TRAIL in Alzheimer's Disease. Dement Geriatr Cogn Dis Extra 5(3):424-34.

Wyss-Coray T. 2006. Inflammation in Alzheimer disease: driving force, bystander or beneficial response? Nat Med 12(9):1005-15.

Wyss-Coray T, Rogers J. 2012. Inflammation in Alzheimer disease-a brief review of the basic science and clinical literature. Cold Spring Harb Perspect Med 2(1):a006346.

Xia MQ, Qin SX, Wu LJ, Mackay CR, Hyman BT. 1998. Immunohistochemical study of the beta-chemokine receptors CCR3 and CCR5 and their ligands in normal and Alzheimer's disease brains. Am J Pathol 153(1):31-7.

Xu DE, Zhang WM, Yang ZZ, Zhu HM, Yan K, Li S, Bagnard D, Dawe GS, Ma QH, Xiao ZC. 2014. Amyloid precursor protein at node of Ranvier modulates nodal formation. Cell Adh Migr 8(4):396-403.

Xu ZQ, Huang H, Chen YL, Gao YY, Xu J, Marshall C, Cai ZY, Xiao M. 2016. Different Expression Patterns of Amyloid-beta Protein Precursor Secretases in Human and Mouse Hippocampal Neurons: A Potential Contribution to Species Differences in Neuronal Susceptibility to Amyloid-beta Pathogenesis. J Alzheimers Dis.

Yan P, Bero AW, Cirrito JR, Xiao Q, Hu X, Wang Y, Gonzales E, Holtzman DM, Lee JM. 2009. Characterizing the appearance and growth of amyloid plaques in APP/PS1 mice. J Neurosci 29(34):10706-14.

Yin KJ, Cirrito JR, Yan P, Hu X, Xiao Q, Pan X, Bateman R, Song H, Hsu FF, Turk J and others. 2006. Matrix metalloproteinases expressed by astrocytes mediate extracellular amyloid-beta peptide catabolism. J Neurosci 26(43):10939-48.

Yu HM, Zhao YM, Luo XG, Feng Y, Ren Y, Shang H, He ZY, Luo XM, Chen SD, Wang XY. 2012. Repeated lipopolysaccharide stimulation induces cellular senescence in BV2 cells. Neuroimmunomodulation 19(2):131-6.

Yuyama K, Sun H, Mitsutake S, Igarashi Y. 2012. Sphingolipid-modulated exosome secretion promotes clearance of amyloid-beta by microglia. J Biol Chem 287(14):10977-89.

Yuyama K, Sun H, Sakai S, Mitsutake S, Okada M, Tahara H, Furukawa J, Fujitani N, Shinohara Y, Igarashi Y. 2014. Decreased amyloid-beta pathologies by intracerebral loading of glycosphingolipid-enriched exosomes in Alzheimer model mice. J Biol Chem 289(35):24488-98.

Yuyama K, Sun H, Usuki S, Sakai S, Hanamatsu H, Mioka T, Kimura N, Okada M, Tahara H, Furukawa J and others. 2015. A potential function for neuronal exosomes: sequestering intracerebral amyloid-beta peptide. FEBS Lett 589(1):84-8.

Zhong Z, Ewers M, Teipel S, Burger K, Wallin A, Blennow K, He P, McAllister C, Hampel H, Shen Y. 2007. Levels of beta-secretase (BACE1) in cerebrospinal fluid as a predictor of risk in mild cognitive impairment. Arch Gen Psychiatry 64(6):718-26.

Zimmermann H. 1994. Signalling via ATP in the nervous system. Trends Neurosci 17(10):420-6.

Zlokovic BV. 2008. The blood-brain barrier in health and chronic neurodegenerative disorders. Neuron 57(2):178-201.

Zou C, Shi Y, Ohli J, Schuller U, Dorostkar MM, Herms J. 2016. Neuroinflammation impairs adaptive structural plasticity of dendritic spines in a preclinical model of Alzheimer's disease. Acta Neuropathol 131(2):235-46.

Chapter 2

MICROGLIA CHANGE FROM A REACTIVE TO AN AGE-LIKE PHENOTYPE WITH THE TIME IN CULTURE

Cláudia Caldeira[1,2], Ana Filipa Oliveira[1], Carolina Cunha[1], Ana Rita Vaz[1], Ana Sofia Falcão[1], Adelaide Fernandes[1,3], Dora Brites[1,3]

[1] Research Institute for Medicines (iMed.ULisboa), Faculdade de Farmácia, Universidade de Lisboa, Lisboa, Portugal;

[2] Centro de Investigação Interdisciplinar Egas Moniz, Egas Moniz – Cooperativa de Ensino Superior, CRL, Campus Universitário, Monte de Caparica, Portugal;

[3] Department of Biochemistry and Human Biology, Faculdade de Farmácia, Universidade de Lisboa, Lisboa, Portugal.

Front Cell Neurosci. **(2014) 8:152**

Acknowledgments

This work was supported by FEDER (COMPETE Programme) and by National funds (FCT - Fundação para a Ciência e a Tecnologia – Project Pest-OE/SAU/UI4013/2011-2013, and PTDC/SAU-FAR/118787/2010 to Dora Brites), the prize Edgar Cruz e Silva 2012 from Grupo de Estudo do Envelhecimento Cerebral e Demência to Dora Brites, and by a grant from Universidade de Lisboa/Fundação Amadeu Dias to Adelaide Fernandes. We thank Dr Ana L. Cardoso and Dr Maria C. Pedroso de Lima for their techinal support and advice with microRNA determination, and André Frederico, Joana Rodrigues and Joana Serrano for techinal assistance. The funding organization had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Abstract

Age-related neurodegenerative diseases have been associated with chronic neuroinflammation and microglia activation. However, cumulative evidence supports that inflammation only occurs at an early stage once microglia change the endogenous characteristics with ageing and switch to irresponsive/senescent and dystrophic phenotypes with disease progression. Thus, it will be important to have the means to assess the role of reactive and aged microglia when studying advanced brain neurodegeneration processes and age-associated related disorders. Yet, most studies are done with microglia from neonates since there are no adequate means to isolate degenerating microglia for experimentation. Indeed, only a few studies report microglia isolation from aged animals, using either short-term cultures or high concentrations of mitogens in the medium, which trigger microglia reactivity. The purpose of this study was to develop an experimental process to naturally age microglia after isolation from neonatal mice and to characterize the cultured cells at 2 days *in vitro* (DIV), 10 DIV and 16 DIV. We found that 2 DIV (young) microglia had predominant amoeboid morphology and markers of stressed/reactive phenotype. In contrast, 16 DIV (aged) microglia evidenced ramified morphology and increased metalloproteinase (MMP)-2 activation, as well as reduced MMP9, glutamate release and nuclear factor kappa-B (NF-κB) activation, in parallel with decreased expression of Toll-like receptor (TLR)-2 and TLR4, capacity to migrate and phagocytose. These findings together with the reduced expression of microRNA (miR)-124, and miR-155, decreased autophagy, enhanced senescence associated β-galactosidase activity and elevated miR-146a expression, are suggestive that 16 DIV cells mainly correspond to irresponsive/senescent microglia. Data indicate that the model represent an opportunity to understand and control microglial ageing, as well as to explore strategies to recover microglia surveillance function.

Keywords: autophagic capacity; *in vitro* cell ageing; microglia; microRNAs; migration; phagocytosis; reactivity; senescence

1. Introduction

Microglia are the first line of defense against brain injury. In the healthy brain microglia actively survey surrounding parenchyma via dynamic movement of processes (Nimmerjahn et al., 2005) and are kept in a relatively quiescent state in part due to specific signals derived from neurons and astrocytes (Cardona et al., 2006; Lyons et al., 2007). Upon brain injury or changes of central nervous system (CNS) homeostasis microglia are capable of acquiring diverse and complex phenotypes, allowing them to participate in the cytotoxic response, immune regulation, and injury resolution. The classical pro-inflammatory M1 phenotype is cytotoxic and release pro-inflammatory cytokines while the M2 polarization contributes to neuroprotection through the release of anti-inflammatory cytokines and growth factors (Chhor et al., 2013; Evans et al., 2013). These transitional phenotypes may exert beneficial or destructive effects depending on the stimuli, their duration and the environment they encounter (Schwartz et al., 2006). Thus, balance between M1 and M2 phenotypes can be considered a desirable therapeutic goal.

Age-related CNS disorders have been related with chronic and progressive neuronal loss but also with chronic mild neuroinflammation involving activated/primed microglia (Maezawa et al., 2011; Williamson et al., 2011). These cells showed to switch from M2 to M1 phenotype with age and disease progression (Solito and Sastre, 2012; Varnum and Ikezu, 2012). However, other studies claim that neuroinflammation is only present in the early stages of Alzheimer´s disease (AD), once lately disappears (Wojtera et al., 2012) and that, instead, microglia become senescent/dystrophic (Graeber and Streit, 2010) and less responsive to stimulation with age (Njie et al., 2012; Streit and Xue, 2012). The dysmorphic characteristics of aged microglia suggested that, rather than maintaining an overactivated state, microglia may display decreased ability to mount a normal response to injury. Indeed, reduced migration (Damani et al., 2011), clearance (Li, 2013) and production of neurotrophic factors (Ma et al., 2013), as well as inability to shift from a pro-inflammatory to an anti-inflammatory state to regulate injury and repair have been observed in aged microglia (Norden and Godbout, 2013) and related with senescence (Streit and Xue, 2012). These changes in microglia potentially contribute to an increased susceptibility and neurodegeneration as a function of age. Accordingly, nonsteroidal anti-inflammatory drugs (NSAIDs) were only successful when administered before the development of neurodegeneration (Weggen et al., 2001). If administered in later stages of disease they showed to be detrimental (Martin et al., 2008), reinforcing that microglia may switch from a reactive to an irresponsive phenotype along the progression of AD and other age-associated CNS disabilities. Restraining of aged microglia may weak even

more the already decreased neuroprotective properties of the cell in removing extracellular protein aggregates. These changes in microglia neuroprotective properties will potentially contribute to enhance neurodegeneration and susceptibilities with ageing and reveal the need of adequate experimental models to follow the changes in microglia performance accordingly to cell senescence.

Most of the work intended to evaluate the neurodegenerative network associated with ageing has used cultures of microglia derived from early postnatal brains, which differ from adult or aged ones (Harry, 2013). Recently, a few studies compared behaviour of microglia isolated from animals at different ages. In these studies young and aged microglia were isolated using a Percol-based method (von Bernhardi et al., 2011; Njie et al., 2012) or distinctly isolated using a mild-trypsinization method for embryonic/neonatal microglia and Percol-based method for adult and aged microglia (Lai et al., 2013). In addition, these cells were analyzed either 24-48 h after isolation (Njie et al., 2012; Lai et al., 2013) or following trypsinization when kept in culture for several weeks in the presence of conditioned medium containing increased levels of mitogens (von Bernhardi et al., 2011). Such methods may promote microglia activation and bias the translation of culture findings, since it has been suggested that microglia may need some time in culture to recover its quiescent state (Cristovão et al., 2010). Moreover, there are no means to isolate degenerating microglia for experimentation (Njie et al., 2012) once only the more resistant ones will survive to the isolation procedure. Nevertheless, the hypothesis of microglia senescence during aging and related neurodegenerative diseases emerged as a key determinant (Luo et al., 2010). *In vitro* ageing of astrocytes and neurons has demonstrated to be associated with different cell response to stimuli, with the younger cells evidencing an increased reactivity when compared to the older ones (Falcão et al., 2005; 2006). In addition, it was shown that the repeated stimulation of the microglia cell line BV2 with lipopolysaccharide (LPS) lead to cell senescence corroborating the idea that sustained neuroinflammation may ultimately contribute to a microglia senescent phenotype (Yu et al., 2012). Therefore, we decided to isolate microglia from neonatal mice and culture cells from 2 days *in vitro* (DIV) until 16 DIV, similarly to what we previously did with neurons and astrocytes, and to explore ageing-related differences in functional response characteristics associated to “young” and “aged” microglia phenotypes. We assessed changes in microglia morphology, nuclear factor kappa-B (NF-κB) signalling pathway activation, Toll-like receptor (TLR) expression, phagocytic ability and migration capacity, as well as cell death, inflammatory microRNA (miRNA) profiling, autophagy and senescence-associated β-galactosidase (SA-β-gal) in mice primary cortical cell cultures maintained up to 16 DIV.

2. Materials and Methods

2.1. Animals

Animal care followed the recommendations of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Council Directive 86/609/EEC) and National Law 1005/92 (rules for protection of experimental animals). All animal procedures were approved by the Institutional animal care and use committee. Every effort was made to minimize the number of animals used and their suffering.

2.2. Primary culture of microglia

Mixed glial cultures were prepared from 1-to-2 day-old CD1 mice as previously described (McCarthy and de Vellis, 1980), with minor modifications (Gordo et al., 2006). Cells (4 x 10^5 cells/cm^2) were plated on uncoated 12-well tissue culture plates (with 18 mm coverslips) or 75-cm^2 culture flasks in culture medium [DMEM-Ham's F-12 medium supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, nonessential amino acids 1X, 10% fetal bovine serum (FBS), and 1% antibiotic-antimycotic solution] and maintained at 37ºC in a humidified atmosphere of 5% CO_2.

Microglia were isolated as previously described by Saura et al. (Saura et al., 2003). Briefly, after 21 days in culture, microglia were obtained by mild trypsinization with a trypsin-EDTA solution diluted 1:3 in DMEM-Ham's F12 for 45 to 60 min. The trypsinization resulted in detachment of an upper layer of cells containing all the astrocytes, whereas the microglia remained attached to the bottom of the well. The medium containing detached cells was removed and the initial mixed glial-conditioned medium was added. Mixed cultures were maintained in culture for 21 days to achieve the maximal yield and purity of the cultures. In fact, astrocyte contamination was less than 2% with 0% of neurons, as assessed by immunocytochemical staining with a primary antibody against GFAP and MAP-2, respectively, followed by a species-specific fluorescent-labeled secondary antibody (Silva et al., 2010).

2.3. Characterization of microglia along the days in culture

After mild trypsinization, attached cells on uncoated 18-mm coverslips were maintained in culture until reaching 2, 10 or 16 days in vitro (DIV) for characterization, with medium replaced every 4 days. Microglia characterization was first performed considering cell morphology and NF-κB activation, at these 3 time-points, and thereafter only at 2 and 16 DIV for additional properties related with migration ability, phagocytic capacity, differential cell reactive ability and markers of cell senescence.

2.4. Cell morphological analysis

For morphological analysis, cells were fixed for 20 min with freshly prepared 4% (w/v) paraformaldehyde in phosphate buffer saline (PBS) and a standard immunocytochemical technique was performed using a primary antibody raised against Iba1 (rabbit, 1:250; Wako Pure Chemical Industries Ltd, Osaka, Japan), and a secondary Alexa Fluor 594 goat anti-rabbit (1:1000; Invitrogen Corporation, Carlsbad, CA, USA). To identify the total number of cells, microglial nuclei were stained with Hoechst 33258 dye. Fluorescence was visualized using an AxioCam HRm camera adapted to an AxioSkope® microscope (Zeiss). Pairs of U.V. and red-fluorescence images of ten random microscopic fields (original magnification: 400X) were acquired per sample. To quantitatively characterize microglia morphology we used the particle measurement feature in ImageJ (1.47v, USA) to automatically measure the 2D area, perimeter, and Feret's diameter of single microglia cells. Feret's (maximum) diameter, a measure of cell length, is the greatest distance between any two points along the cell perimeter. We also evaluated the transformation index, first defined by Fujita and co-workers as [perimeter of cell (μm)]^2/4π [cell area (μm^2)], which categorizes microglia ramification status (Fujita et al., 1996). A cell with long processes and a small soma exhibits a large index that is dependent on cell shape but independent of cell size.

2.5. Detection of NF-κB activation

For immunofluorescence detection of NF-κB nuclear translocation, cells were fixed as above and a standard indirect immunocytochemical technique was carried out using a polyclonal rabbit anti-p65 NF-κB subunit antibody (1:200; Santa Cruz Biotechnology®, CA, USA) as the primary antibody and an anti-rabbit Cy2 as the secondary antibody (1:1000; GE Healthcare, Chalfont St. Giles, UK). Microglial nuclei were stained with Hoechst 33258 dye. Fluorescence was visualized and acquired as above. NF-κB positive nuclei were identified by localization of the NF-κB p65 subunit staining exclusively at the nucleus and total cells were counted to determine the percentage of NF-κB-positive nuclei at each cell DIV group.

2.6. Determination of cell death

We used phycoerythrin-conjugated annexin V (annexin V-PE) and 7-amino-actinomycin D (7-AAD) (Guava Nexin® Reagent, #4500-0450, Millipore) to determine the percentage of viable, early-apoptotic and late-apoptotic/necrotic cells by flow cytometry. After incubation adherent microglia were collected by trypsinization and added to the cells present in the incubation media. After centrifugation cells were resuspended in PBS containing 1% bovine serum albumin (BSA), stained with annexin

V-PE and 7-AAD, following manufacturer's instructions, and analyzed on a Guava easyCyte 5HT flow cytometer (Guava Nexin® Software module, Millipore), as previously described (Barateiro et al., 2012). Three populations of cells can be distinguished in this assay: viable cells (annexin V-PE and 7-AAD negative), early apoptotic cells (annexin V-PE positive and 7-AAD negative), and late stages of apoptosis or dead cells (annexin V-PE and 7-AAD positive).

2.7. Assessement of microglia migration

Cell migration assays were performed in a 48-well microchemotaxis Boyden chamber (Neuro Probe, Gaithersburg, MD, USA), as previously described (Miller and Stella, 2009) with minor modifications. The bottom wells, filled with ATP (10 µM), a known chemoattractant for microglia migration, served as positive controls. The 8 µm diameter polycarbonate membranes with polyvinylpyrrolidone (PVP) surface treatment was equilibrated in control medium and after chamber set up, 50 µl of cell suspension containing 2 x 10^4 cells was added to each top well. After 6 h incubation in a CO_2 incubator at 37ºC for microglial migration, membrane was fixed with cold methanol and cells stained with 10% Giemsa in PBS. Non-migrated cells on the upper side of the membrane were wiped off with a filter wiper. The rate of migration was determined by counting cells on the lower membrane surface in 10 microscopic fields to cover all the well, acquired using a Leica DFC490 camera adapted to an AxioSkope HBO50 microscope. For each experiment, at least three wells per condition were analyzed.

2.8. Evaluation of phagocytic properties of microglia

To evaluate the phagocytic capacity of the primary microglial cultures, cells collected at 2 and 16 DIV were incubated with 0.0025% (w/w) of 1 µm fluorescent latex beads (Sigma Chemical Co., St. Louis, MO, USA) for 75 min at 37ºC and fixed with freshly prepared 4% (w/v) paraformaldehyde in PBS. Microglia were stained for Iba1, nuclei counterstained with Hoechst dye, and fluorescence was visualized and acquired as above. The number of ingested beads per cell was counted. Results are presented as mean number of ingested beads per cell and as the percentage of cells that phagocytosed <5, 5-10 or >10 beads.

2.9. Determination of supplementary features of microglia reactive ability

We used several markers to assess microglia reactive ability, such as the concentration of glutamate and the activation of MMP2 and MMP9 in the extracellular media, together with the expression of TLR2, TLR4, miR-124 and miR-155.

Glutamate content in the media derived from microglial cultures was determined as described before (Silva et al., 2012) by an adaptation of the L-glutamic acid kit (Roche), using a 10-fold volume reduction. The reaction was performed in a 96-well microplate and the absorbance measured at 490 nm. A calibration curve was used for each assay. All samples and standards were analyzed in duplicate and the mean value was used.

Detection of MMPs activity was performed as previously mentioned (Silva et al., 2010). Aliquots of culture supernatant were analyzed by SDS-PAGE zymography in 0.1% gelatine–10% acrylamide gels under non-reducing conditions. After electrophoresis, gels were washed for 1 h with 2.5% Triton X-100 (in 50 mM Tris pH7.4; 5 mM $CaCl_2$; 1 μM $ZnCl_2$) to remove SDS and renature the MMP species in the gel. Then the gels were incubated in the developing buffer (50 mM Tris pH7.4; 5 mM $CaCl_2$; 1 μM $ZnCl_2$) overnight to induce gelatine lysis. For enzyme activity analysis, the gels were stained with 0.5% Coomassie Brilliant Blue R-250 and destained in 30% ethanol/10% acetic acid/H_2O. Gelatinase activity, detected as a white band on a blue background, was quantified by computerized image analysis and normalized with total cellular protein.

TLR2 and TLR4 mRNA expression was performed by RealTime PCR as usual in our laboratory (Barateiro et al., 2013). Total RNA was extracted from microglia using TRIzol® (LifeTechnologies), according to manufacturer's instructions. Total RNA was quantified using Nanodrop ND-100 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Aliquots of 0.5 μg of total RNA were treated with DNase I and then reverse transcribed cDNA using oligo-dT primers and SuperScript II Reverse Transcriptase under the recommended conditions. Quantitative RealTime PCR (qRT-PCR) was performed using β-actin as an endogenous control to normalize the expression level of TLR2 and TLR4 transcription factors. The following sequences were used as primers: TLR2 sense 5'-TGCTTTCCTGCTGAAGATTT-3' and anti-sense 5'-TGTACCGCAACAGCTTCAGG-3'; TLR4 sense 5'-ACCTGGCTGGTTTACACGTC-3' and anti-sense 5'-GTGCCAGAGACATTGCAGAA-3'; β-actin sense 5'-GCTCCGGCATGTGCAA-3' and anti-sense 5'-AGGATCTTCATGAGGTAGT-3'. qRT-PCR was performed on a 7300 Real time PCR System (Applied Biosystems) using a SYBR Green qPCR Master Mix (Fermentas). The PCR was performed in 96 well plates with each sample performed in triplicate, and no-template control was included for each amplificate. qRT-PCR was performed under optimized conditions: 94ºC at 3 min followed by 40 cycles at 94ºC for 0.15 min, 62ºC for 0.2 min and 72ºC for 0.15 min. In order to verify the specificity of the amplification, a melt-curve analysis was performed, immediately after the amplification protocol. Non-specific products of PCR were not found in any case. Relative mRNA concentrations were calculated using the Pfaffl modification of the ΔΔCT equation (CT, cycle number at

which fluorescence passes the threshold level of detection), taking into account the efficiencies of individual genes. The results were normalized to β-actin in the same sample and the initial amount of the template of each sample was determined as relative expression by the formula 2-ΔΔCT. ΔCT is a value obtained, for each sample, by the difference between the mean CT value of each gene and the mean CT value of β-actin. ΔΔCT of one sample is the difference between its ΔCT value and ΔCT of the sample chosen as reference, in our case 2 DIV cells.

Expression of miR-124 and miR-155, which has been related with microglia activation phenotype, was performed by RT-PCR. Total RNA was extracted from primary microglia cultures using the miRCURY™ Isolation Kit – Cells (Exiqon), according to the manufacturer's recommendations for cultured cells. Briefly, after cell lysis, the total RNA was adsorbed to a silica matrix, washed with the recommended buffers an eluted with 35 µl RNase-free water by centrifugation. After RNA quantification, cDNA conversion for miRNA quantification was performed with the universal cDNA Synthesis Kit (Exiqon) using 20 ng total RNA according to the following protocol: 60 min at 42ºC followed by heat-inactivation of the reverse transcriptase for 5 min at 95ºC. qRT-PCR was performed in an iQ5 thermocycler (Applied Biosystems) using 96-well plates. For miRNA quantification the miRCURY LNA™ Universal RT microRNA PCR system (Exiqon) was used in combination with pre-designed primers (Exiqon) for miR- miR146a and sRNA U6 (reference gene). The reaction conditions consisted of polymerase activation/denaturation and well-factor determination at 95ºC for 10 min, followed by 50 amplification cycles at 95ºC for 10 s and 60ºC for 1 min (ramp-rate 1.6º/s). The miRNA fold change with respect to 2 DIV cells was determined by the Pfaffl method, taking into consideration different amplification efficiencies of miRNAs in all experiments. The amplification efficiency for each target was determined according to the formula: $E = 10^{(-1/S)} - 1$, where S is the slope of the obtained standard curve.

2.10. Assessment of microglia senescence

Microglia senescence was evaluated by determining the activity of SA-β-gal, expression of miR-146a and capacity to undergo autophagy. Microglial SA-β-gal activity was determined using the Cellular senescence assay kit (Millipore), according to the manufacturer instructions. Microglial nuclei were counterstained with hematoxylin. Brightfield microscopy images of ten random microscopic fields were acquired per sample. The number of turquoise stained microglia (SA-β-gal-positive cells) was counted to determine the percentage of senescent cells.

To confirm the senescent status of microglia it was also assessed the expression of the senescence-related miR-146a by RT-PCR. Total RNA was extracted and expression

of miR-146a was assayed using pre-designed primers (Exiqon) for miR-146a and sRNA U6 (reference gene) as described above.

Autophagy was determined by both immunocytochemistry of microtubule-associated-protein-light-chain-3 (LC3) punctate and Western Blot detection of LC3 and Beclin 1 bands. For immunocytochemistry, cells were fixed as above and standard immunocytochemical technique was performed using a primary antibody raised against LC3 protein (rabbit, 1:500; Cell Signaling Technology Inc., MA, USA), and a secondary Alexa Fluor 488 goat anti-rabbit antibody (1:1000; Invitrogen Corporation, CA, USA). To identify the total number of cells, microglial nuclei were stained with Hoechst 33258 dye. Fluorescence was visualized and images acquired as above. The method is based on the increased localization of LC3 autophagosomes when autophagy is induced. Thus the punctate fluorescence produced by LC3 staining provides a sensitive and specific indicator of autophagy (Aoki et al., 2008). Microglial cells presenting LC3 punctate were counted and the percentage of LC3 punctate-positive cells relatively to total microglia was determined. In addition, autophagy was further confirmed by detection of LC3-II, which is associated with autophagic vesicles (Kabeya et al., 2000), and Beclin 1 bands by Western Blot as usual in our laboratory (Barateiro et al., 2012). Cells were washed in ice-cold PBS, lysed in a buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na2EDTA, 1 mM ethylene glycoltetraacetic acid, 1% (v/v) Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 µg/mL leupeptine and 1 mM phenylmethylsulfonyl fluoride, and sonicated for 20 s. The lysate was centrifuged at 14,000 g for 10 min at 4ºC and the supernatants were collected and stored at -80ºC. Protein concentrations were determined using BioRad protein assay (BioRad). Cell extracts containing equal amounts of protein (50 µg) were separated on sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membranes were blocked with 5% non-fat milk, incubated with the primary antibody overnight at 4ºC [rabbit anti-LC3B (1:1000) (#2775, Cell Signaling), mouse anti-Beclin 1 (1:500) (#MABC34, MerckMillipore) or mouse anti-β-actin (1:5,000) (Sigma)], and then with a horseradish peroxidase-labelled secondary antibody for 1 h at room temperature. After extensive washes, immunoreactive bands were detected by LumiGLO® (Cell Signalling, Beverly, MA, USA) and visualized by autoradiography with Hyperfilm ECL. Results were normalized to β-actin expression and expressed as fold vs. vehicle-treated cells.

2.11. Statistical analysis

Significant differences between the parameters evaluated were determined by the two-tailed Student's *t-test* performed on the basis of equal and unequal variance, as

appropriate. Comparison of more than two groups (microglia morphology, NF-kB activation) was done by ANOVA using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA) followed by multiple comparisons Bonferroni *post-hoc* correction. *p* value less than 0.05 were considered statistically significant.

3. Results

3.1. *In vitro* ageing changes microglia morphology to a more ramified cell shape

Phenotypic changes in microglia are often accompanied by a morphological transformation, which has been widely used to categorize different activation states. In general, ramified quiescent microglia changes to an activated state displaying larger somata and shorter, coarser cytoplasmic processes progressing to a full amoeboid morphology (Fujita et al., 1996; Kozlowski and Weimer, 2012). Interestingly, microglia isolated from adult and aged animals show a propensity to acquire a more ramified morphology with thicker and more extensive processes (Lai et al., 2013), indicative of a less activated phenotype with age. So, we started by characterizing microglia morphology at 2, 10 and 16 DIV, following immunollabeling with the cell-specific marker Iba1. As shown in **Figure 2.1**, diverse morphological forms of microglia may be observed throughout cell culturing. The microglial cells at 2 DIV were almost exclusively amoeboid, most frequently evidencing an ovoid shape with a few cells presenting fusiform shape **(Figure 2.1A)**. At 10 DIV, microglia evidence a more heterogeneous morphology with an increased number of cells showing a ramified morphology, bearing typically one or two large processes or a single large lamellipodia, together with larger amoeboid forms **(Figure 2.1B)**. Microglia cultures at 16 DIV still exhibited distinct polarized populations showing rod-like microglia, bipolar microglia with shorter processes and the residual amoeboid cells **(Figure 2.1C)**. To quantitatively evaluate the effect of age on microglia morphology we measured the area, perimeter, and Feret's maximum diameter of microglia **(Figure 2.1D-F)**. Consistent with a transformation of amoeboid to microglia ramified forms, the area, perimeter and the Feret's maximum diameter significantly increased at 16 DIV (~2.0-fold, ~1.6-fold and ~1.6-fold, respectively, $p < 0.05$). Analysis of the transformation index value, a dimensionless number that reflects the degree of process extension, revealed a continuum of the microglial phenotype between the amoeboid and the ramified morphologies **(Figure 2.1G)**. While younger cultures with a predominant amoeboid microglia shape present a low transformation index, older cultures with a more heterogeneous morphological repertoire involving cells with amoeboid and ramified morphologies, displayed an increased transformation index (~1.6-fold, $p < 0.05$).

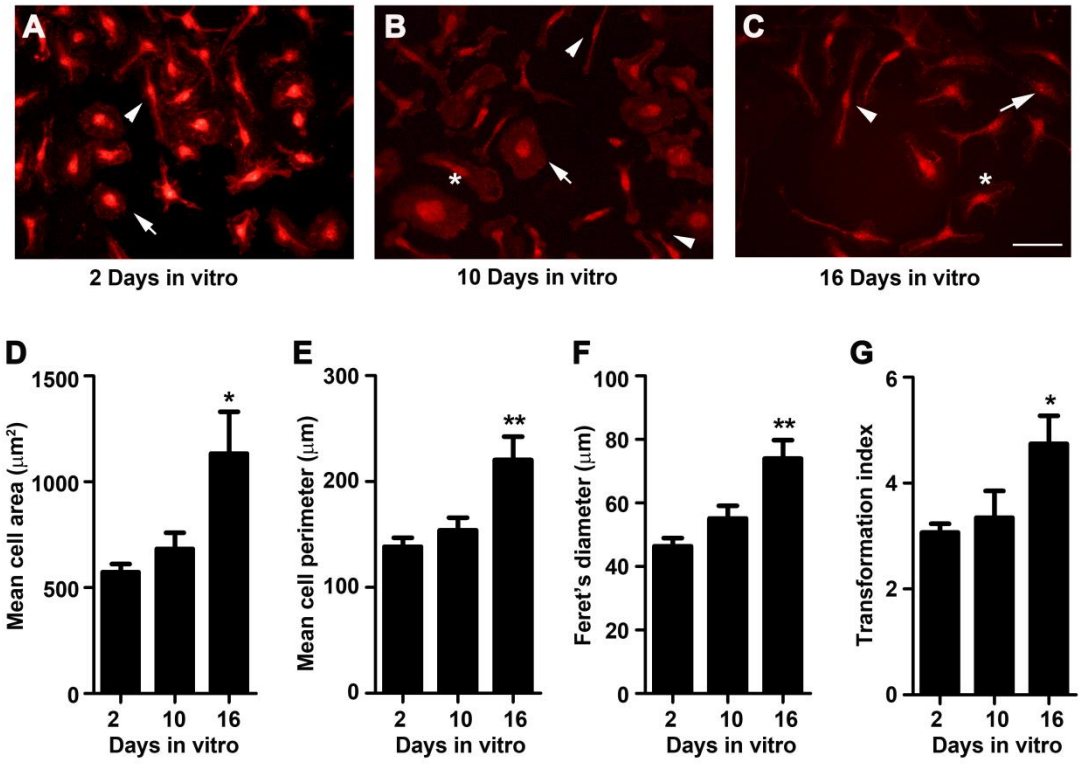

Figure 2. 1 – Microglia morphology changes from amoeboid to a more ramified shape with cell ageing in culture. Microglial cells were kept in culture for 2, 10 and 16 days *in vitro* (DIV), immunostained for Iba1 and their morphology analyzed. **(A)** At 2 DIV, microglia were amoeboid with ovoid shape (arrow) and only a few showed a ramified bipolar morphology (arrowhead). **(B)** At 10 DIV, microglia became more heterogeneous with more cells presenting a ramified morphology (arrowhead), bearing a single large lamellipodia (*) and some a larger amoeboid shape (arrowhead). **(C)** At 16 DIV cells exhibited distinct polarized populations including ramified rod-like microglia (arrowhead), bipolar microglia with shorter processes (*) and residual amoeboid cells (arrow). Microglia area **(D),** perimeter **(E)** and Feret's diameter **(F)** values were measured using the computer program ImageJ; transformation index values **(G)** were calculated as [perimeter of cell (μm)]^2/4π [cell area (μm^2)]. Cultures, n = 4 per group. *Post-hoc* Bonferroni test *p < 0.05, **p < 0.01 *vs.* 2 DIV cells. Each value represents the mean ± SEM. Scale bar equals 50 μm.

3.2. *In vitro* ageing reduces microglia NF-κB activation

Microglia play key immune-related duties, intervening through the production of anti-inflammatory compounds and trophic factors, by phagocytosing non-functional cell and debris, but also by releasing pro-inflammatory cytokines, depending on the stimuli. Production of several cytokines during microglial activation process is associated with the activation of the inducible transcription factor NF-κB (O'Neill and Kaltschmidt, 1997). To explore whether microglia morphological changes along the time in culture could be related with the cell activation state, we investigated NF-κB transactivation at the time

points used to assess morphological alterations. Following microglia immunollabeling for p65 NF-κB subunit, we determined the number of NF-κB-positive nuclei as an indicator of its activation **(Figure 2.2)**. Our results show that microglia express maximal NF-κB activation at 2 DIV decreasing significantly thereafter and reaching minimal levels at 16 DIV (~0.4-fold *vs.* 2 DIV, $p < 0.01$). These results corroborate the previous data in cell morphology and reinforce that microglia are highly reactive at 2 DIV but reduce their activation profile to a minimum state at 16 DIV. Thus, to settle that microglia at these *in vitro* stages may be associated to activated (2 DIV) and to age-like irresponsive cells (16 DIV), we additionally explored several markers that have been associated to age-related alterations in the dynamic behaviour of microglia.

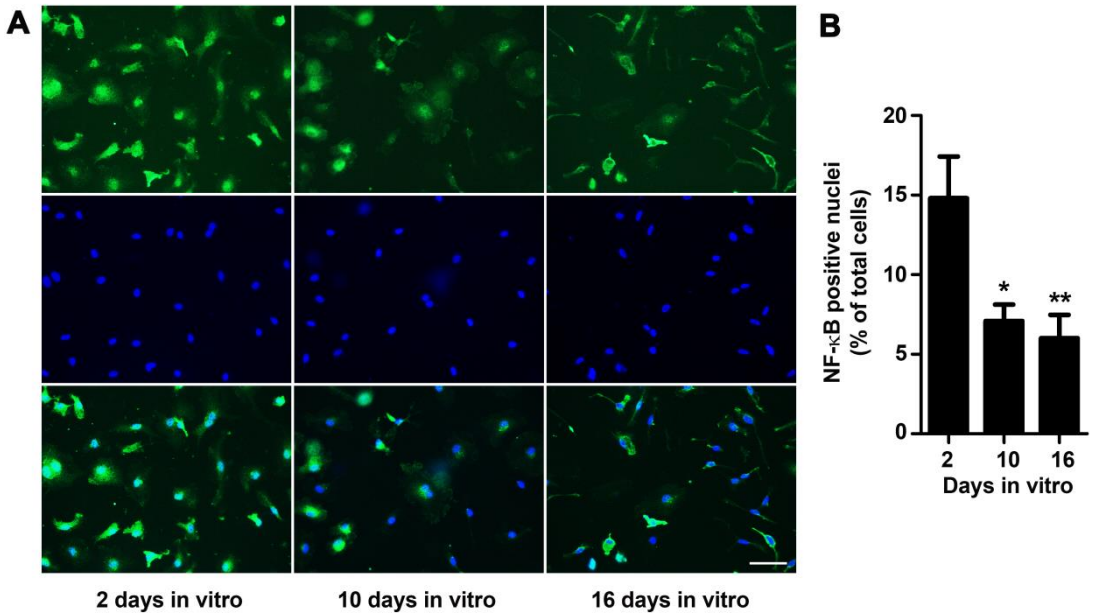

Figure 2. 2 – NF-κB activation decreases with microglia ageing in culture. Microglial cells were kept in culture for 2, 10 and 16 days *in vitro* (DIV), immunostained for NF-κB (green) and their nuclei stained with Hoechst dye (blue). **(A)** Representative images at 2, 10 and 16 DIV. **(B)** Cells bearing a NF-κB-positive nuclei were counted and results expressed in graph bars as mean ± SEM. Cultures, $n = 4$ per group. *Post-hoc* Bonferroni test *$p < 0.05$, **$p < 0.01$ *vs.* 2 DIV cells. Scale bar equals 50 µm.

3.3. Aged microglia show a residual migration ability

Microglia directed migration towards regions of injury, also known as chemotaxis, is a property that seems to be more related to the classically (M1) and alternatively activated microglia (M2a) (Lively and Schlichter, 2013). The release of chemotactic molecules upon brain damage, such as ATP, was indicated to participate in the recruitment of microglia toward lesion sites (Miller and Stella, 2009; Kettenmann et al., 2011). Nevertheless, it was reported that microglia respond to ATP regardless of their activation state (Lively and Schlichter, 2013). Hence, we evaluated the ability of 2 and

16 DIV microglia to migrate towards 10 µM ATP. As shown in **Figure 2.3**, 16 DIV microglia revealed a poor ability to migrate to ATP when compared to 2 DIV cells (~0.1-fold, $p < 0.01$). This finding points to a 2 DIV population of reactive microglia with capacity to migrate to local brain injury in contrast to the aged cells that lose invasion capacity property.

3.4. Aged microglia show reduced phagocytic ability

Microglia are considered the professional phagocytes of the CNS, a function that is crucial along brain development, as well as in pathology and regeneration (Kettenmann et al., 2011). Therefore, and based on the previous results, we hypothesized that ageing in culture could also have adverse effects on microglia phagocytic properties. As expected, 16 DIV microglia showed reduced engulfment ability when compared to 2 DIV cells **(Figure 2.4A)**. Indeed, the average number of beads phagocytosed by each microglial cell was markedly reduced from 2 to 16 DIV (~0.5-fold, $p < 0.01$). In addition, we observed that aged microglia function less effectively than the 2 DIV cells based on the increased number of cells that engulf a small number of beads ($p < 0.01$) together with a decreased ability to digest 5 or more beads ($p < 0.05$) **(Figure 2.4B)**. Altogether these data suggest that *in vitro* ageing of microglia obtained from neonatal mice change their dynamic behavior to a more inert or irresponsive phenotype compatible with an irresponsive/senescent cell.

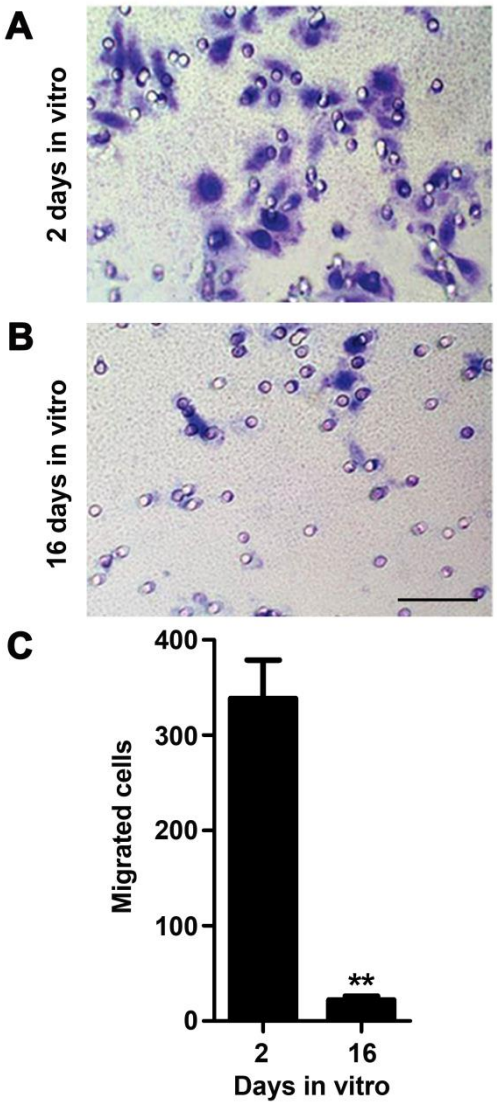

Figure 2. 3 – Microglia migration ability decrease with cell ageing in culture. Microglial cells were kept in culture for 2 and 16 days *in vitro* (DIV) and then cellular chemotactic migration to 10 µM ATP was evaluated using the Boyden chamber method. Representative images of 2 **(A)** and 16 **(B)** DIV microglia that migrated towards ATP were visualized by Giemsa staining. Number of migrated cells was counted and results expressed in graph bars as mean ± SEM (C). Cultures, $n = 4$ per group. *t*- test $^{**}p < 0.01$ *vs.* 2 DIV cells. Scale bar equals 50 µm.

3.5. Microglia retain viability during *in vitro* ageing

Given our previous results we wondered whether the loss of microglia function by *in vitro* ageing was a consequence of reduced cell viability. Therefore, we evaluated microglia cell death by flow cytometry following staining with annexin V-PE and 7-AAD, to differentiate the total amount of cells (adherent plus detached) into viable, early apoptotic and late apoptotic/necrotic cells. As shown in **Table 2.1**, we did not observe differences in cell death between the 2 and the 16 DIV microglia, confirming that changes

in aged microglia response are not due to reduced viability but rather derive from a switch in cellular phenotype and its properties.

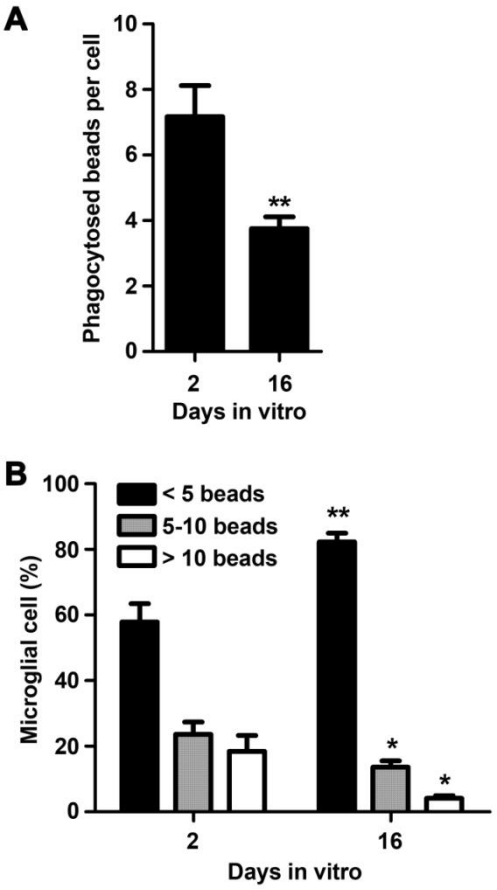

Figure 2. 4 – Microglia phagocytic ability decrease with cell ageing in culture. Microglial cells were kept in culture for 2 and 16 days *in vitro* and then exposed to fluorescent beads to measure their phagocytic capacity. Number of phagocytized beads per cell **(A)** and the number of microglia phagocytosing < 5, 5-10 and > 10 beads **(B)** was counted and results expressed in graph bars as mean ± SEM. Cultures, *n* = 4 per group. *t*- test and *post-hoc* Bonferroni test **p* < 0.05, ***p* < 0.01 *vs.* 2 DIV cells.

Table 2. 1 – Viability of culturing microglia

	Viable	**Early-apoptosis**	**Late-apoptosis/ necrosis**
2 DIV	81.8 (±2.6)	9.7 (±0.3)	6.2 (±2.2)
16 DIV	81.7 (±3.0)	11.4 (±1.9)	7.4 (±1.6)

All results are means ± SEM from at least four independent experiments. Microglial were kept in culture for 2 and 16 days in vitro (DIV). The percentage of viable microglia and microglia in early- or late-apoptosis/necrosis was determined by flow cytometer with phycoerythrin-conjugated annexin V (annexin V-PE) and 7-amino-actinomycin D (7-AAD). The three populations were distinguished as follows: viable cells (annexin V-PE and 7-AAD negative), early apoptotic cells (annexin V-PE positive and 7-AAD negative), and cells in late stages of apoptosis or dead cells (annexin V-PE and 7-AAD positive).

3.6. Supplementary features of microglia reactive ability are reduced in aged cells

Since 16 DIV microglia have shown decreased ability to respond to chemotactic signals or to phagocytose extracellular particles, features that were not related with loss of cell survival **(Table 2.1)**, we next decided to evaluate whether microglia aged in culture would also present additional markers of reduced reactive ability. Glutamate was shown to be released by activated microglia (Noda et al., 1999; Barger et al., 2007; Takaki et al., 2012), so we decided to evaluate the extracellular content of glutamate. As depicted in **Figure 2.5A**, 16 DIV microglia showed to release lower levels of glutamate to the culture media than the 2 DIV cells (~0.7-fold, $p < 0.01$). Interestingly, when evaluating MMP2 and MMP9 activation in the extracellular media we verified that the influence of ageing was also notorious **(Figure 2.5B)**. Indeed we observed a marked increase of MMP2 (~2.2-fold, $p < 0.05$) and a decrease of MMP9 (~0.6-fold, $p < 0.05$) in the aged microglia when compared to 2 DIV cells. Again, the expression of TLR2 and TLR4 that is associated with microglia activation (Banks and Robinson, 2010; Liu et al., 2012) very much decreased in the 16 DIV microglia (~0.4-fold, $p < 0.01$, **Figure 2.5C**). Recently, immune regulation by miR-124 was indicated to downregulate microglia activation (Ponomarev et al., 2011) in contrast with miR-155 that was shown to have a pro-inflammatory role in microglia (Cardoso et al., 2012), to be related with the M1 phenotype (Ponomarev et al., 2013) and to be up-regulated upon activation (Lu et al., 2011). Corroborating previous findings, the decreased expression of both miR-124 and miR-155 in 16 DIV microglia, as compared to 2 DIV cells (~0.5- and 0.4-fold, respectively, $p < 0.01$), further reinforce that the cells become irresponsive/senescent when maintained in culture.

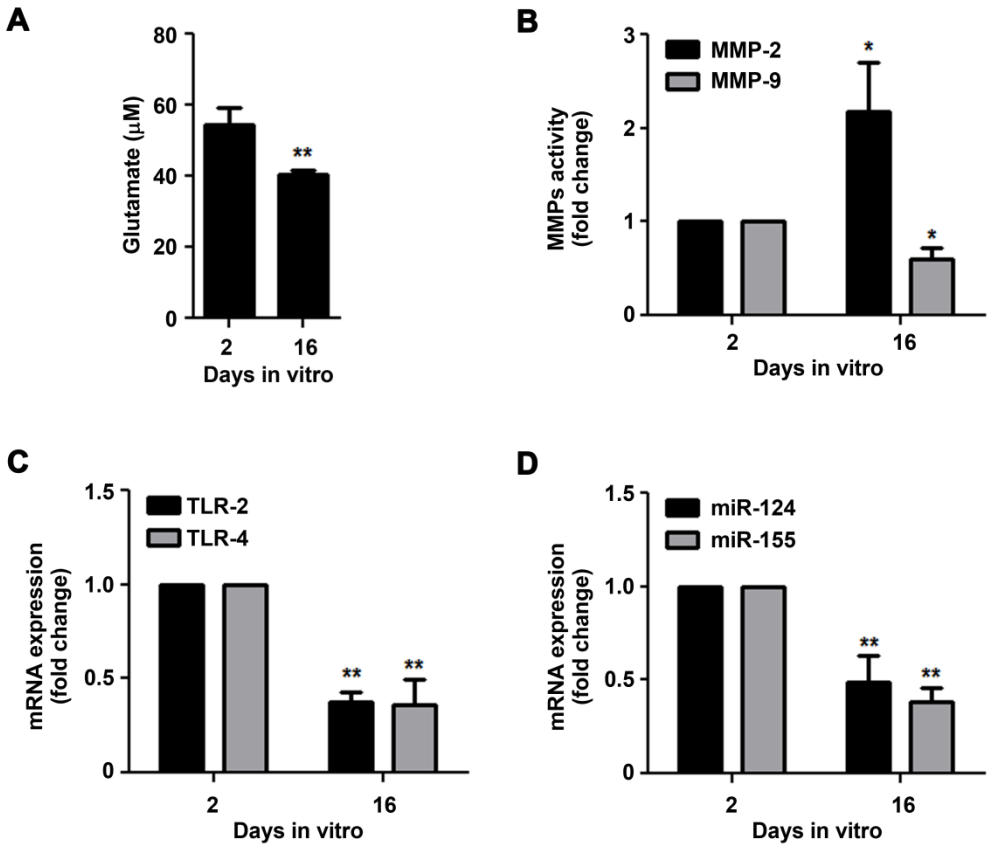

Figure 2. 5 – Microglia supplementary features of reactive ability are reduced in aged cells. Microglial cells were kept in culture for 2 and 16 days *in vitro* (DIV). **(A)** Extracellular glutamate levels were determined using a commercial kit. **(B)** Metalloproteinases (MMP)-2 and MMP9 activities assessed by gelatin zymography. Expression of TLR2 and TLR4 **(C)**, as well as of miR-124 and miR-155 **(D)** was evaluated by RealTime PCR. Results are expressed in graph bars as mean ± SEM. Cultures, $n = 4$ per group. *t*-test $*p < 0.05$, $**p < 0.01$ *vs.* 2 DIV cells.

3.7. 16 DIV microglia show common markers of senescence

Senescent microglia have been described to become dysfunctional and less efficient in their neuroprotective effects during ageing in the human brain and in AD (Streit and Xue, 2012; Krabbe et al., 2013). The main proposal of the present study was to obtain an experimental model able to reproduce irresponsive/senescent microglia and be used for exploring detrimental effects by ageing and associated-neurodegenerative diseases. As so, we decided to evaluate if the *in vitro* aged microglia displayed typical signs of cell senescence. The senescence phenotype has been associated with changes in cellular morphology, increased activity for SA-β-gal, permanent DNA damage, chromosomal instability and altered inflammatory secretome (Sikora et al., 2011). More recently, new biomarkers of age-associated senescence have been reported including an increased expression of miR-146a in aged macrophages (Jiang et al., 2012) and a reduced

capacity to undergo autophagy (Ma et al., 2011). Quantitative assay of SA-β-gal activity revealed that the percentage of positively stained cells markedly increased from 2 to 16 DIV (~2.5-fold, $p < 0.01$), as evidenced in **Figure 2.6A-B**. Similarly, we noticed a significant elevation in the expression of miR-146a along the cell ageing in culture (~2.3-fold, $p < 0.05$, **Figure 2.6C**). Finally, we evaluated autophagic capacity by LC3 immunostaining. As it may be observed in **Figure 2.7A**, 2 DIV cells display an increased amount of LC3 punctates when compared to 16 DIV microglia. Counting of LC3 punctate-positive cells confirmed that a reduced number of 16 DIV cells were undergoing autophagy (~0.7-fold, $p < 0.05$, **Figure 2.7B**). Next we evaluated the expression of LC3-II that is formed through lipidation of LC3-I during autophagy (Kabeya et al., 2000) and we additionally determined the Beclin-1 protein, recognized to have a central role in such process (Kang et al., 2011), by Western Blot **(Figure 2.7C-D)**. Our results clearly show that LC3-II and Beclin-1 are markedly reduced in in 16 DIV microglia when compared to 2 DIV cells (~0.4-fold and ~0.3-fold, respectively, $p <0.01$), confirming a reduced autophagy by the aged microglia.

Overall, our data indicate that primary microglia harvested from neonatal mouse pups first evidence an increased reactive ability changing to an irresponsive/senescent cell when maintained in culture. Aged cells evidence a reduced ability to become activated, to migrate and to phagocytose, in parallel with markers of cellular senescence. Therefore, this *in vitro* model can be very useful in the exploitation of microglia reactivity and irresponsiveness to stimuli, respectively. In addition, changes in microglia miRNA signature may constitute a precious help in evaluating the key role of microglia as a determinant in age-associated CNS disorders and in modulating microglia dynamic properties.

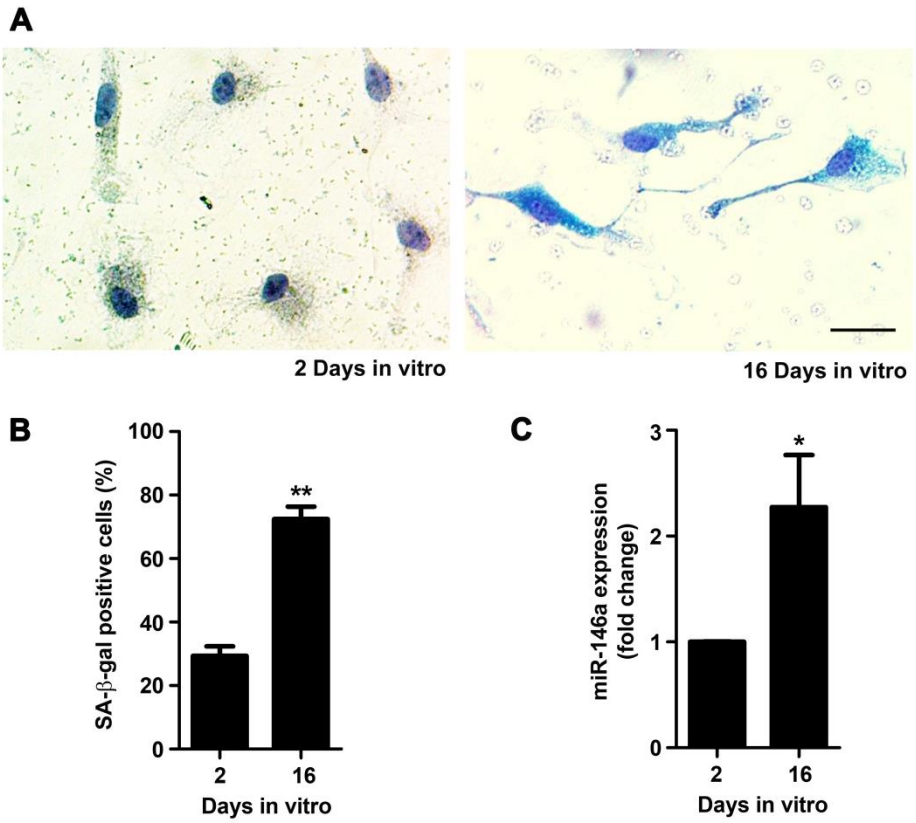

Figure 2. 6 – Microglia aged in culture display signs of senescence, including increased senescent-associated β-galactosidase (SA-β-gal) activity and miR-146a expression. Microglial cells were kept in culture for 2 and 16 days *in vitro* (DIV). Activity of SA-β-gal was determined using a commercial kit. **(A)** Representative images of 2 and 16 DIV microglia showing SA-β-gal staining. **(B)** SA-β-gal-positive cells were counted and results expressed in graph bars as mean ± SEM. **(C)** miR-146a expression was evaluated by RealTime PCR. Results are expressed in graph bars as mean ± SEM. Cultures, $n = 4$ per group. *t*-test $^{*}p < 0.05$, $^{**}p < 0.01$ *vs.* 2 DIV cells. Scale bar equals 20 µm.

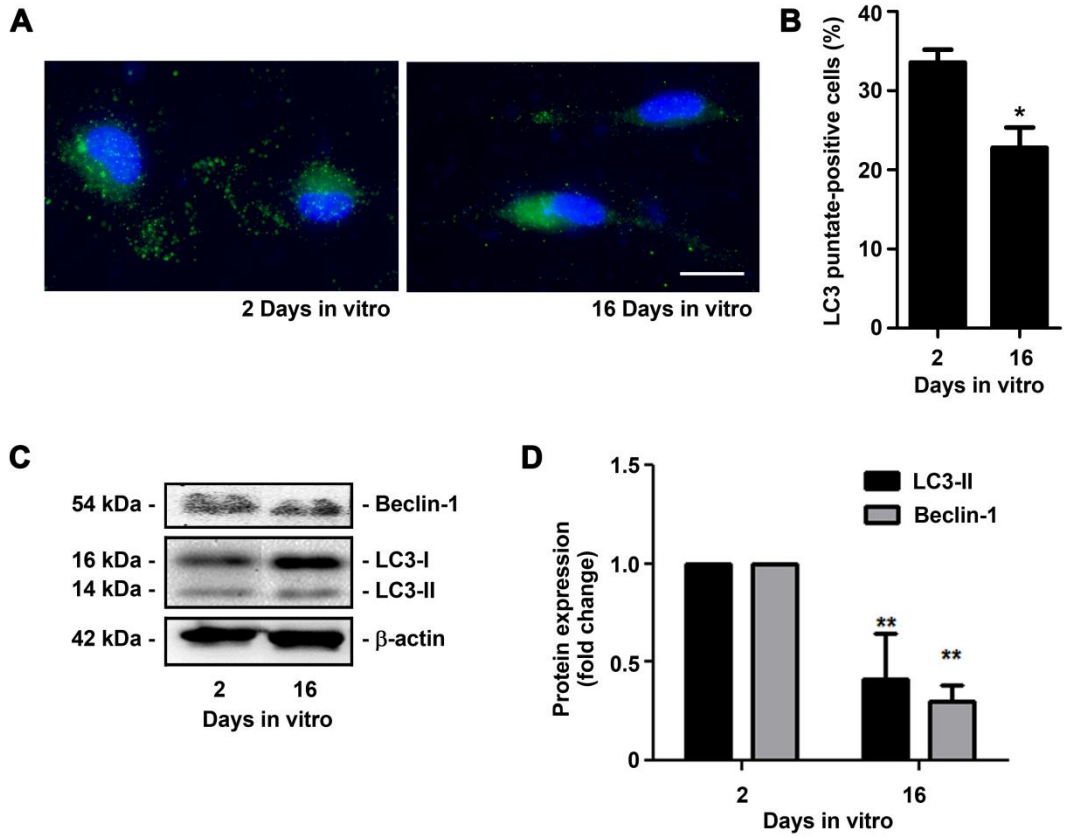

Figure 2. 7 – Microglia aged in culture show reduced autophagic capacity. Microglial cells were kept in culture for 2 and 16 days *in vitro* (DIV), immunostained for LC3 and total cell lysates were analyzed for the presence of LC3-II and Beclin-1. **(A)** Representative images of 2 and 16 DIV microglia showing LC3 punctates. **(B)** Cells displaying LC3 punctates were counted and results expressed in graph bars as mean ± SEM. **(C)** LC3-I/LC3-II and Beclin 1 protein expression was assessed by Western Blot and **(D)** densitometric analysis is expressed in graph bars as mean ± SEM. Cultures, $n = 4$ per group. *t*-test $*p < 0.05$, $**p < 0.01$ *vs.* 2 DIV cells. Scale bar equals 20 µm.

4. Discussion

Experiments in this study set out to investigate age-specific differences in the dynamic functional profiles of neonatal microglia aged in culture, from 2 DIV up to 16 DIV. Here we show that microglia isolated from neonatal pups evidence markers of reactive ability at early time culture changing their phenotype along *in vitro* culture to less responsive cells that present senescence biomarkers and miRNA profiling characteristic of microglia deactivation. Collectively, our results indicate that microglia ageing can be reproduced *in vitro* using long-term murine cultures, which may be used as a model to evaluate microglia performance in age-associated disorders, inasmuch due to the similar characteristics such cells evidence to human microglia (Torres-Platas et al., 2014).

Mouse primary neonatal microglial cultures have the advantage to more closely represent their *in situ* counterparts when compared to immortalized cells, although by growing in isolation lack the normal CNS microenvironment (Ni and Aschner, 2010). Indeed, primary cultured microglia are not oncogene immortalized and are differentiated in mixed glial cultures before isolation. The protocol here described originates microglial cultures that exceed 97% purity and has been used as a model for activated CNS-resident microglia (Carson et al., 1998; Schmid et al., 2009) and to prepare polarized M1 and M2 phenotypes (Jang et al., 2013). Indeed, it was previously suggested that the isolation process is a sufficient stimulus to induce microglia activation (Cristovão et al., 2010). There is a high controversy on whether neonatal microglia are less (Moussaud and Draheim, 2010) or more reactive than adult (Christensen et al., 2014) and aged cells (Njie et al., 2012). Discrepancies also exist in the scientific community based on studies that consider microglia overactivation and increased release of pro-inflammatory cytokines with age and neurodegenerative diseases (for review see (Wong, 2013; Mosher and Wyss-Coray, 2014), in contrast with others evidencing dystrophic microglia and senescence (Streit et al., 2004; Streit and Xue, 2013), decreased phagocytosis (Floden and Combs, 2011; Li, 2013), lower reactivity to stimulation (Damani et al., 2011; Njie et al., 2012), delayed response to exogenous ATP and decreased microglial process motility (Hefendehl et al., 2014). Such contradictory results may be caused by different experimental sets and conditions. Moreover, most of the data were derived from experimental models using LPS-induced microglia activation, when it is well known that only a small amount is able to enter the brain parenchyma (Banks and Robinson, 2010). Therefore, the effects of peripheral immunostimulation by intravenously administered LPS dose are indirect and some of them mediated by the cells that comprise the BBB. Another important aspect to consider is that nonsteroidal anti-inflammatory drugs were only successful when administered before the development of neurodegeneration (Weggen et al., 2001). When administered in later stages of disease they showed to be detrimental (Martin et al., 2008). These findings may underlie a first proinflammatory stage in neurodegenerative diseases associated to neuroinflammmation, later switching to dysfunctional neurodegeneration associated with a loss of microglia dynamic properties. Indeed, neither the typical inflammatory nor the anti-inflammatory phenotypes were identified at end-stage amyotrophic lateral sclerosis (Nikodemova et al., 2013) and microglial dystrophy associated with their senescence (Flanary, 2005), as well as to aged and AD brain (Lopes et al., 2008).

Lack of knowledge on the molecular mechanisms implicated in aged microglia dysfunction and how it is related to an increased individual vulnerability to neurodegenerative diseases has hindered the development of effective therapy for

preventing or even halting the CNS network degenerative process. Major problems to investigate such mechanisms are determined by the current *in vitro* microglial models using cell lines, primary microglia isolated from neonatal murine animals and *ex vivo* isolation from adult and aged brain. First models are not suitable for the research of neurodegenerative diseases where ageing is crucial since long-term culture experiments are critical, and the last one only provides specific microglia subsets that resist to the isolation procedure (Moussaud and Draheim, 2010; von Bernhardi et al., 2011) or that are separated based on immunomagnetic cell sorting steps (Cardona et al., 2006). However, mixed microglial populations may coexist in the CNS and were also shown to be developed in culture (Szabo and Gulya, 2013; Gertig and Hanisch, 2014). In addition, microglia functionality from adult and aged animals is not well preserved, the yield is low and the cells undergo extensive cell death resulting in activation of the surviving population (von Bernhardi et al., 2011). The *in vitro* model we developed to obtain microglia senescence in primary culture has been used to identify ageing-associated changes in fibroblasts at the molecular level (Chen et al., 2009). Finally, we have not used microglia culturing with astrocytes to avoid the complex interactions between these cells (Tanaka et al., 1999) that would be a disadvantage to assess natural microglia maturity and senescence. Therefore, establishment of well-defined stable in vitro cultures freshly isolated from neonatal mice and characterization of microglial phenotype with the time in culture may provide advantages over the other methods to determine ageing microglial dynamics modifications and therapeutic approaches to recover microglial functionality.

Microglia morphology changed along *in vitro* maintenance from an almost exclusive round amoeboid shape to distinct polarized populations, including an increased number of ramified cells. In accordance, mixed primary glial cultures from embryonic rats have previously showed the existence of cells with an amoeboid morphology in the early stages of *in vitro* differentiation, which changed to mixed populations of amoeboid and ramified cell morphologies in older cultures (Szabo and Gulya, 2013). Interestingly, data from microglia isolated from different age animals also corroborate such findings with adult microglia presenting a more ramified morphology, in contrast with an amoeboid shape of embryonic and neonatal microglia (Lai et al., 2013). This is in line with *in vivo* data indicating that invading neonatal microglia have a predominant rounded morphology that differentiate with time into a surveying phenotype characterized by a small soma and highly branched processes (Hanisch and Kettenmann, 2007). Our aged microglia cultures besides exhibiting ramified and amoeboid cells also presented cells with a bipolar shape and shorter large processes. Morphological signs of microglia senescence with ageing were observed *in vivo* and defined as abnormal morphological

features, such as shortened, gnarled, beaded, or fragmented cytoplasmic processes, and loss of fine ramifications and formation of spheroidal swellings (Streit et al., 2004). Therefore, we hypothesize that such cells with shortened processes represent microglia with less ability to become reactive and should include a relevant population of senescent microglia.

The morphological changes of in vitro ageing microglia occurred in parallel with a decrease in the transactivation of NF-κB. It is well known that this transcription factor is found throughout the cytoplasm, translocating to the nucleus upon activation triggering the transcription of target genes, such as the pro-inflammatory cytokines (O'Neill and Kaltschmidt, 1997). Therefore, maximal activation of NF-κB two days after isolation is consistent with an inflammatory phenotype that shifts to a deactivated microglia along with the time in culture. Intriguingly, although we showed a decreased NF-κB activation at 16 DIV, the activation of this transcription factor has been associated with the ageing process. A recent report has shown that hypothalamic microglial NF-κB activation promoting a residual inflammatory status is required for systemic ageing (Zhang et al., 2013). Nevertheless, a marked down-regulation of NF-κB was also observed in cultured senescent human WI-38 fibroblasts (Helenius et al., 1996). Considering that activators of the NF-κB signalling pathway are determinants of inflammation and ageing process (Balistreri et al., 2013) and that CNS inflammation is present in the early stages of age-related disorders such as AD but disappears with disease progression (Streit et al., 2009), our *in vitro* aged microglia may represent a dystrophic and irresponsive phenotype whose functions have progressively declined as recently observed in mice with AD-like pathology (Krabbe et al., 2013).

The reduced migration observed for 16 DIV cells is in line with recent data showing that aged microglia become less dynamic with slower acute responses and lower rates of process motility (Damani et al., 2011). Here we measured ATP-induced microglial chemotaxis, which occurs via P2X4R and P2Y12R purinergic receptors (Ohsawa et al., 2007). Interestingly, even considering that the expression of P2X4R in microglia is not age-dependent, the P2Y12R expression varies with animal age increasing to a maximum at 6-8 months and decreasing thereafter to extremely low levels at 13-15 months (Lai et al., 2013). Thus, it is possible that our aged microglia present reduced expression of purinergic receptors which may be in the origin of the reduced ability to migrate to ATP. Moreover, since it was demonstrated that monocyte chemoattractant protein-1 (MCP-1) that is produced downstream NF-κB activation is involved in the migration of microglia (Deng et al., 2009), based on the age-dependent reduction of NF-κB nuclear

translocation we have observed it is reasonable to consider that the MCP-1-dependent migration pathway may also be affected.

Phagocytosis is crucial to maintain tissue homeostasis and innate immune balance, by ingesting both foreign pathogens and autologous apoptotic cells (Napoli and Neumann, 2009). Infectious pathogens are phagocytosed through TLRs or complement receptors to elicit the release of pro-inflammatory cytokines (Napoli and Neumann, 2009), while apoptotic cells or cellular debris are internalized through phosphatidylserine receptors, integrins or TREM2 to trigger immunosuppressive signaling with the release of anti-inflammatory cytokines (Li, 2012). During ageing, clearance of both foreign pathogens and autologous apoptotic cells is diminished and has been associated with immunosenescence (Li, 2013). In accordance, microglia from aged mice also internalized less amyloid-β peptide (Aβ) than microglia from neonatal or young mice (Njie et al., 2012), corroborating our findings that 16 DIV microglia have a reduced ability to phagocytose possibly due to the manifestation of a senescent phenotype. Interestingly, microglial cells maintained in mixed primary neuronal-glial co-cultures were shown to phagocytose more when amoeboid than in the ramified form, a property that decreased during culturing (Szabo and Gulya, 2013). In agreement, we observed a shift to a more ramified phenotype with cell ageing, which paralleled a reduced phagocytic ability.

Activated microglia were shown to release increased levels of glutamate (Noda et al., 1999; Barger et al., 2007; Takaki et al., 2012). However, several studies have shown lower glutamate concentration in older subjects when compared to younger individuals (Kaiser et al., 2005; Sailasuta et al., 2008; Chang et al., 2009) and an age-dependent decline of glutamate release in mice (Minkeviciene et al., 2008). This finding is in line with the reduced glutamate levels we obtained in aged cell cultures. Similarly, the increased activation of MMP2 we observed in 16 DIV microglia was identified in senescent cells (Liu and Hornsby, 2007; Lu et al., 2009; Malaquin et al., 2013). In what concerns MMP9 there is some discrepancy between authors. Some indicate increased activity with age (Simpson et al., 2013) and others a decrease (Bonnema et al., 2007; Paczek et al., 2008), as we obtained. Furthermore, we think that the marked reduced expression we obtained at 16 DIV microglia for TLR2 and TLR4 (0.5-fold and 0.4-fold, respectively), as compared to 2 DIV cells, define with no doubt that 16 DIV microglia will be less able to respond to LPS immunostimulation. Actually, TLR4 that is critical for the recognition of LPS, as well as TLR2 that also recognizes some LPS species, are inducers of microglia activation leading to the production of proinflammatory cytokines (Banks and Robinson, 2010; Liu et al., 2012). Curiously, the TLR4 downregulation-

mediated supression of TNF-α and lL-1β expression revealed to also be accompanied by the suppression of NF-κB (Yao et al., 2013).

MicroRNAs (miRNAs) are an abundant class of highly evolutionarily conserved small noncoding RNAs that are involved in posttranscriptional gene silencing, regulating diverse biological processes (Ambros, 2004). miR-146a was first associated with the innate immune response as a negative feedback regulator in TLR signaling (Taganov et al., 2006), and more recently implicated in age-related dysfunction of macrophages (Jiang et al., 2012). Our results clearly showed that aged microglia express increased levels of miR-146a, thus corroborating their senescent phenotype. Interestingly, expression of miR-146a that has been associated with several neurodegenerative disorders (Sinha et al., 2011; Jiang et al., 2013), was found elevated in the aged mouse (Jiang et al., 2012; Olivieri et al., 2013), in the cerebrospinal fluid of AD patients (Alexandrov et al., 2012), and to be induced in microglia upon Aβ and inflammatory challenge (Li et al., 2011). As so, our *in vitro* old microglia reproduce the ageing-associated phenotype encountered in in late-life common disorders. Moreover, decreased miR-124 and miR-155 that revealed a negative correlation with age (Fichtlscherer et al., 2010; Noren Hooten et al., 2010; Smith-Vikos and Slack, 2012), parallelled by the enhanced miR-146a expression, further reinforce that 16 DIV microglia mainly represent aged-like microglia. In addition, the reduced miR-124 obtained in these cells, indicated as being associated to the M2a-alternatively activated state (Freilich et al., 2013) and to inhibit inflammation (Prinz and Priller, 2014), strengthen their dormant/senescent phenotype. In contrast, the predominant amoeboid morphology together with increased NF-κB activation, cell migration, phagocytosis and the higher levels of miR-155 expression in 2 DIV microglia, as compared with aged cells, are indicative of a major representation of cells with a stressed/reactive phenotype. Indeed, a strong up-regulation of miR-155 expression was shown to have a pro-inflammatory role in microglia (Cardoso et al., 2012) and to drive the M1 phenotype (Ponomarev et al., 2013) corroborating the stressful properties of 2 DIV cultured cells.

Nowadays, changed morphology and increased activity of SA-β-gal of permanently growth arrested cells are considered cellular senescence markers (Sikora et al., 2011). In accordance, 16 DIV microglia displayed a marked increase of SA-β-gal activity when compared to 2 DIV cells. The activity of SA-β-gal was also associated with senescence-unrelated settings, such as contact inhibition or serum starvation (Severino et al., 2000). Nevertheless, as observed by the Iba1 pictures, our microglia culture did not reach confluence and was not cultured under serum starvation, attesting that the increase of

SA-β-gal activity results from a senescent phenotype. Indeed, decreased microglia migration, phagocytic ability, NF-κB activation and increased SA-β-gal, as we here observed, have been indicated as hallmarks of microglial ageing and cell senescence (Mosher and Wyss-Coray, 2014).

Several neurodegenerative diseases are characterized by the formation of intracellular protein aggregates in affected brain regions, indicating a failure of protein degradation system (McCray and Taylor, 2008). Autophagy is a stress-induced catabolic process responsible for the degradation of long-lived proteins and damaged organelles (Levine and Klionsky, 2004) that was shown to decline with ageing (Bergamini, 2006) and to determine cell and individual lifespan (Juhasz et al., 2007). A study using the senescence accelerated mouse prone 8, a rodent model of ageing and senile dementia, showed a reduced autophagic activity by ageing with long-lasting autophagosomes and increased LC3 expression (Ma et al., 2011). In accordance, affected neurons with abnormal autophagosomes (Lee, 2009) and impaired of autophagy (Komatsu et al., 2006) were seen in neurodegeneration. We showed that 16 DIV microglia display a reduced amount of LC3 punctates suggestive of a decreased formation of autophagossomes. This finding was further corroborated by the decrease we also observed in the expression of Beclin 1 in the aged cells. Beclin 1 is known to intervene from autophagosome formation to autophagosome/endosome maturation but to also have other additional functions (Kang et al., 2011). Interestingly, Beclin 1 was recently considered to be required for efficient phagocytosis and to be reduced in microglia isolated from AD brains (Lucin et al., 2013), thus accounting to explain the reduced phagocytic ability in our 16 DIV cells and to such impairment in mice with AD-like pathology (Krabbe et al., 2013).

It is worth mentioning that the 2 DIV and 16 DIV microglia differently react to some tested neurotoxins, as we anticipated. We used unconjugated bilirubin that has previously shown to induce the release of the pro-inflammatory cytokines TNF-α and IL-1β from astrocytes and microglia in concentrations similar to those induced by 10 ng/ml LPS (Fernandes et al., 2004; Gordo et al., 2006; Brites et al., 2009), and Aβ at 50 nM, a concentration that was indicated to trigger microglia activation (Maezawa et al., 2011). The test was first directed to the expression of the high-mobility group box protein-1 (HMGB1) a mediator of inflammation directly correlated with NK-κB protein activation (Rovina et al., 2013). Both stimuli enhanced cellular HMGB1 expression in 2 DIV microglia (80 and 100% increase for bilirubin and Aβ respectively; results not shown), without affecting the 16 DIV cells. Next, and similarly to what we have obtained for

HMGB1, up-regulation of mRNA levels of IL-18 expression capable of more potently induce inflammatory response than IL-1β (Alboni et al., 2010) was again associated with the young/reactive microglia treated with bilirubin (60% increase over control) or Aβ (>100% increase over control) (data not shown), but no alterations were noticed in the aged cells.

5. Conclusions

Overall, we demonstrate that microglia isolated from neonatal mice and kept in vitro in long-term cultures switch from an activated/reactive phenotype to cells presenting ageing-like alterations. Our results show that in vitro aged microglia changes their morphology to a more ramified shape, with a reduced basal NF-κB activation, impaired migration and phagocytic abilities, low TLR2 and TLR4 expression, as well as reduced MMP9 and glutamate efflux. This study is the first to provide the inflamma-miRNA signature for microglia ageing in primary cultures. The cells evidenced decreased expression of miR-155 and miR-124, reduced autophagic capacity, and increased miR-146a expression and SA-β-gal activity, consistent with the existence of senescent cells at 16 DIV in culture. In conclusion, given the phenotypical changes observed for young/reactive and irresponsive/senescent microglia along the time in culture, the *in vitro* model of microglia ageing could be of interest to assess how different signals may diversely modify cell functionality in separate microglia populations and to link increased age with risk for neurodegenerative diseases and other age-related phenomena.

6. References

Alboni, S., Cervia, D., Sugama, S., and Conti, B. (2010). Interleukin 18 in the CNS. *J Neuroinflammation* 7**,** 9. doi: 10.1186/1742-2094-7-91742-2094-7-9

Alexandrov, P.N., Dua, P., Hill, J.M., Bhattacharjee, S., Zhao, Y., and Lukiw, W.J. (2012). microRNA (miRNA) speciation in Alzheimer's disease (AD) cerebrospinal fluid (CSF) and extracellular fluid (ECF). *Int J Biochem Mol Biol* 3**,** 365-373.

Ambros, V. (2004). The functions of animal microRNAs. *Nature* 431**,** 350-355. doi: 10.1038/nature02871nature02871

Aoki, H., Kondo, Y., Aldape, K., Yamamoto, A., Iwado, E., Yokoyama, T., Hollingsworth, E.F., Kobayashi, R., Hess, K., Shinojima, N., Shingu, T., Tamada, Y., Zhang, L., Conrad, C., Bogler, O., Mills, G., Sawaya, R., and Kondo, S. (2008). Monitoring autophagy in glioblastoma with antibody against isoform B of human microtubule-associated protein 1 light chain 3. *Autophagy* 4**,** 467-475.

Balistreri, C.R., Candore, G., Accardi, G., Colonna-Romano, G., and Lio, D. (2013). NF-kappaB pathway activators as potential ageing biomarkers: targets for new therapeutic strategies. *Immun Ageing* 10**,** 24. doi: 10.1186/1742-4933-10-24

Banks, W.A., and Robinson, S.M. (2010). Minimal penetration of lipopolysaccharide across the murine blood-brain barrier. *Brain Behav Immun* 24**,** 102-109. doi: 10.1016/j.bbi.2009.09.001

Barateiro, A., Miron, V.E., Santos, S.D., Relvas, J.B., Fernandes, A., Ffrench-Constant, C., and Brites, D. (2013). Unconjugated bilirubin restricts oligodendrocyte differentiation and axonal myelination. *Mol Neurobiol* 47**,** 632-644. doi: 10.1007/s12035-012-8364-8

Barateiro, A., Vaz, A.R., Silva, S.L., Fernandes, A., and Brites, D. (2012). ER Stress, Mitochondrial Dysfunction and Calpain/JNK Activation are Involved in Oligodendrocyte Precursor Cell Death by Unconjugated Bilirubin. *Neuromolecular Med*. doi: 10.1007/s12017-012-8187-9

Barger, S.W., Goodwin, M.E., Porter, M.M., and Beggs, M.L. (2007). Glutamate release from activated microglia requires the oxidative burst and lipid peroxidation. *J Neurochem* 101**,** 1205-1213. doi: 10.1111/j.1471-4159.2007.04487.x

Bergamini, E. (2006). Autophagy: a cell repair mechanism that retards ageing and age-associated diseases and can be intensified pharmacologically. *Mol Aspects Med* 27**,** 403-410. doi: 10.1016/j.mam.2006.08.001

Bonnema, D.D., Webb, C.S., Pennington, W.R., Stroud, R.E., Leonardi, A.E., Clark, L.L., Mcclure, C.D., Finklea, L., Spinale, F.G., and Zile, M.R. (2007). Effects of age on plasma matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs). *J Card Fail* 13**,** 530-540. doi: 10.1016/j.cardfail.2007.04.010

Brites, D., Fernandes, A., Falcão, A.S., Gordo, A.C., Silva, R.F., and Brito, M.A. (2009). Biological risks for neurological abnormalities associated with hyperbilirubinemia. *J Perinatol* 29 Suppl 1**,** S8-13. doi: 10.1038/jp.2008.214

Cardona, A.E., Huang, D., Sasse, M.E., and Ransohoff, R.M. (2006). Isolation of murine microglial cells for RNA analysis or flow cytometry. *Nat Protoc* 1**,** 1947-1951. doi: 10.1038/nprot.2006.327

Cardoso, A.L., Guedes, J.R., Pereira De Almeida, L., and Pedroso De Lima, M.C. (2012). miR-155 modulates microglia-mediated immune response by down-regulating SOCS-1 and promoting cytokine and nitric oxide production. *Immunology* 135**,** 73-88. doi: 10.1111/j.1365-2567.2011.03514.x

Carson, M.J., Reilly, C.R., Sutcliffe, J.G., and Lo, D. (1998). Mature microglia resemble immature antigen-presenting cells. *Glia* 22**,** 72-85. doi: 10.1002/(SICI)1098-1136(199801)22:1<72::AID-GLIA7>3.0.CO;2-A

Chang, L., Jiang, C.S., and Ernst, T. (2009). Effects of age and sex on brain glutamate and other metabolites. *Magn Reson Imaging* 27**,** 142-145. doi: 10.1016/j.mri.2008.06.002

Chen, Y., Johansson, E., Fan, Y., Shertzer, H.G., Vasiliou, V., Nebert, D.W., and Dalton, T.P. (2009). Early onset senescence occurs when fibroblasts lack the glutamate-cysteine ligase modifier subunit. *Free Radic Biol Med* 47**,** 410-418. doi: 10.1016/j.freeradbiomed.2009.05.003

Chhor, V., Le Charpentier, T., Lebon, S., Ore, M.V., Celador, I.L., Josserand, J., Degos, V., Jacotot, E., Hagberg, H., Savman, K., Mallard, C., Gressens, P., and Fleiss, B. (2013). Characterization of phenotype markers and neuronotoxic potential of polarised primary microglia in vitro. *Brain Behav Immun* 32**,** 70-85. doi: 10.1016/j.bbi.2013.02.005

Christensen, L.B., Woods, T.A., Carmody, A.B., Caughey, B., and Peterson, K.E. (2014). Age-related differences in neuroinflammatory responses associated with a distinct profile of regulatory markers on neonatal microglia. *J Neuroinflammation* 11**,** 70. doi: 10.1186/1742-2094-11-70

Cristovao, A.C., Saavedra, A., Fonseca, C.P., Campos, F., Duarte, E.P., and Baltazar, G. (2010). Microglia of rat ventral midbrain recovers its resting state over time in vitro: let microglia rest before work. *J Neurosci Res* 88**,** 552-562. doi: 10.1002/jnr.22219

Damani, M.R., Zhao, L., Fontainhas, A.M., Amaral, J., Fariss, R.N., and Wong, W.T. (2011). Age-related alterations in the dynamic behavior of microglia. *Aging Cell* 10**,** 263-276. doi: 10.1111/j.1474-9726.2010.00660.x

Deng, Y.Y., Lu, J., Ling, E.A., and Kaur, C. (2009). Monocyte chemoattractant protein-1 (MCP-1) produced via NF-kappaB signaling pathway mediates migration of amoeboid microglia in the periventricular white matter in hypoxic neonatal rats. *Glia* 57**,** 604-621. doi: 10.1002/glia.20790

Evans, M.C., Couch, Y., Sibson, N., and Turner, M.R. (2013). Inflammation and neurovascular changes in amyotrophic lateral sclerosis. *Mol Cell Neurosci* 53**,** 34-41. doi: 10.1016/j.mcn.2012.10.008

Falcão, A.S., Fernandes, A., Brito, M.A., Silva, R.F.M., and Brites, D. (2005). Bilirubin-induced inflammatory response, glutamate release, and cell death in rat cortical astrocytes are enhanced in younger cells. *Neurobiol Dis* 20**,** 199-206. doi: 10.1016/j.nbd.2005.03.001

Falcão, A.S., Fernandes, A., Brito, M.A., Silva, R.F.M., and Brites, D. (2006). Bilirubin-induced immunostimulant effects and toxicity vary with neural cell type and maturation state. *Acta Neuropathol* 112**,** 95-105. doi: 10.1007/s00401-006-0078-4

Fernandes, A., Silva, R.F., Falcão, A.S., Brito, M.A., and Brites, D. (2004). Cytokine production, glutamate release and cell death in rat cultured astrocytes treated with unconjugated bilirubin and LPS. *J Neuroimmunol* 153**,** 64-75. doi: 10.1016/j.jneuroim.2004.04.007

Fichtlscherer, S., De Rosa, S., Fox, H., Schwietz, T., Fischer, A., Liebetrau, C., Weber, M., Hamm, C.W., Roxe, T., Muller-Ardogan, M., Bonauer, A., Zeiher, A.M., and Dimmeler, S. (2010). Circulating microRNAs in patients with coronary artery disease. *Circ Res* 107**,** 677-684. doi: 10.1161/CIRCRESAHA.109.215566

Flanary, B. (2005). The role of microglial cellular senescence in the aging and Alzheimer diseased brain. *Rejuvenation Res* 8**,** 82-85. doi: 10.1089/rej.2005.8.82

Floden, A.M., and Combs, C.K. (2011). Microglia demonstrate age-dependent interaction with amyloid-beta fibrils. *J Alzheimers Dis* 25**,** 279-293. doi: 10.3233/JAD-2011-101014

Freilich, R.W., Woodbury, M.E., and Ikezu, T. (2013). Integrated expression profiles of mRNA and miRNA in polarized primary murine microglia. *PLoS One* 8**,** e79416. doi: 10.1371/journal.pone.0079416

Fujita, H., Tanaka, J., Toku, K., Tateishi, N., Suzuki, Y., Matsuda, S., Sakanaka, M., and Maeda, N. (1996). Effects of GM-CSF and ordinary supplements on the ramification of microglia in culture: a morphometrical study. *Glia* 18**,** 269-281. doi: 10.1002/(SICI)1098-1136(199612)18:4<269::AID-GLIA2>3.0.CO;2-T

Gertig, U., and Hanisch, U.K. (2014). Microglial diversity by responses and responders. *Front Cell Neurosci* 8**,** 101. doi: 10.3389/fncel.2014.00101

Gordo, A.C., Falcão, A.S., Fernandes, A., Brito, M.A., Silva, R.F., and Brites, D. (2006). Unconjugated bilirubin activates and damages microglia. *J Neurosci Res* 84**,** 194-201. doi: 10.1002/jnr.20857

Graeber, M.B., and Streit, W.J. (2010). Microglia: biology and pathology. *Acta Neuropathol* 119**,** 89-105. doi: 10.1007/s00401-009-0622-0

Hanisch, U.K., and Kettenmann, H. (2007). Microglia: active sensor and versatile effector cells in the normal and pathologic brain. *Nat Neurosci* 10**,** 1387-1394. doi: 10.1038/nn1997

Harry, G.J. (2013). Microglia during development and aging. *Pharmacol Ther* 139**,** 313-326. doi: 10.1016/j.pharmthera.2013.04.013

Hefendehl, J.K., Neher, J.J., Suhs, R.B., Kohsaka, S., Skodras, A., and Jucker, M. (2014). Homeostatic and injury-induced microglia behavior in the aging brain. *Aging Cell* 13**,** 60-69. doi: 10.1111/acel.12149

Helenius, M., Hanninen, M., Lehtinen, S.K., and Salminen, A. (1996). Changes associated with aging and replicative senescence in the regulation of transcription factor nuclear factor-kappa B. *Biochem J* 318 (Pt 2)**,** 603-608.

Jang, E., Lee, S., Kim, J.H., Seo, J.W., Lee, W.H., Mori, K., Nakao, K., and Suk, K. (2013). Secreted protein lipocalin-2 promotes microglial M1 polarization. *FASEB J* 27**,** 1176-1190. doi: 10.1096/fj.12-222257

Jiang, M., Xiang, Y., Wang, D., Gao, J., Liu, D., Liu, Y., Liu, S., and Zheng, D. (2012). Dysregulated expression of miR-146a contributes to age-related dysfunction of macrophages. *Aging Cell* 11**,** 29-40. doi: 10.1111/j.1474-9726.2011.00757.x

Jiang, W., Zhang, Y., Meng, F., Lian, B., Chen, X., Yu, X., Dai, E., Wang, S., Liu, X., Li, X., and Wang, L. (2013). Identification of active transcription factor and miRNA regulatory pathways in Alzheimer's disease. *Bioinformatics* 29**,** 2596-2602. doi: 10.1093/bioinformatics/btt423btt423

Juhasz, G., Erdi, B., Sass, M., and Neufeld, T.P. (2007). Atg7-dependent autophagy promotes neuronal health, stress tolerance, and longevity but is dispensable for metamorphosis in Drosophila. *Genes Dev* 21**,** 3061-3066. doi: 10.1101/gad.1600707

Kabeya, Y., Mizushima, N., Ueno, T., Yamamoto, A., Kirisako, T., Noda, T., Kominami, E., Ohsumi, Y., and Yoshimori, T. (2000). LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *EMBO J* 19**,** 5720-5728. doi: 10.1093/emboj/19.21.5720

Kaiser, L.G., Schuff, N., Cashdollar, N., and Weiner, M.W. (2005). Age-related glutamate and glutamine concentration changes in normal human brain: 1H MR spectroscopy study at 4 T. *Neurobiol Aging* 26**,** 665-672. doi: 10.1016/j.neurobiolaging.2004.07.001

Kang, R., Zeh, H.J., Lotze, M.T., and Tang, D. (2011). The Beclin 1 network regulates autophagy and apoptosis. *Cell Death Differ* 18**,** 571-580. doi: 10.1038/cdd.2010.191

Kettenmann, H., Hanisch, U.K., Noda, M., and Verkhratsky, A. (2011). Physiology of microglia. *Physiol Rev* 91**,** 461-553. doi: 10.1152/physrev.00011.2010

Komatsu, M., Waguri, S., Chiba, T., Murata, S., Iwata, J., Tanida, I., Ueno, T., Koike, M., Uchiyama, Y., Kominami, E., and Tanaka, K. (2006). Loss of autophagy in the central nervous system causes neurodegeneration in mice. *Nature* 441**,** 880-884. doi: 10.1038/nature04723

Kozlowski, C., and Weimer, R.M. (2012). An automated method to quantify microglia morphology and application to monitor activation state longitudinally in vivo. *PLoS One* 7**,** e31814. doi: 10.1371/journal.pone.0031814

Krabbe, G., Halle, A., Matyash, V., Rinnenthal, J.L., Eom, G.D., Bernhardt, U., Miller, K.R., Prokop, S., Kettenmann, H., and Heppner, F.L. (2013). Functional impairment of microglia coincides with Beta-amyloid deposition in mice with

Alzheimer-like pathology. *PLoS One* 8**,** e60921. doi: 10.1371/journal.pone.0060921

Lai, A.Y., Dibal, C.D., Armitage, G.A., Winship, I.R., and Todd, K.G. (2013). Distinct activation profiles in microglia of different ages: a systematic study in isolated embryonic to aged microglial cultures. *Neuroscience* 254**,** 185-195. doi: 10.1016/j.neuroscience.2013.09.010

Lee, J.A. (2009). Autophagy in neurodegeneration: two sides of the same coin. *BMB Rep* 42**,** 324-330. Doi: 10.5483/BMBRep.2009.42.6.324

Levine, B., and Klionsky, D.J. (2004). Development by self-digestion: molecular mechanisms and biological functions of autophagy. *Dev Cell* 6**,** 463-477. doi: 10.1016/S1534-5807(04)00099-1

Li, W. (2012). Eat-me signals: keys to molecular phagocyte biology and "appetite" control. *J Cell Physiol* 227**,** 1291-1297. doi: 10.1002/jcp.22815

Li, W. (2013). Phagocyte dysfunction, tissue aging and degeneration. *Ageing Res Rev* 12**,** 1005-1012. doi: 10.1016/j.arr.2013.05.006

Li, Y.Y., Cui, J.G., Dua, P., Pogue, A.I., Bhattacharjee, S., and Lukiw, W.J. (2011). Differential expression of miRNA-146a-regulated inflammatory genes in human primary neural, astroglial and microglial cells. *Neurosci Lett* 499**,** 109-113. doi: 10.1016/j.neulet.2011.05.044

Liu, D., and Hornsby, P.J. (2007). Senescent human fibroblasts increase the early growth of xenograft tumors via matrix metalloproteinase secretion. *Cancer Res* 67**,** 3117-3126. doi: 10.1158/0008-5472.CAN-06-3452

Liu, T., Gao, Y.J., and Ji, R.R. (2012). Emerging role of Toll-like receptors in the control of pain and itch. *Neurosci Bull* 28**,** 131-144. doi: 10.1007/s12264-012-1219-5

Lively, S., and Schlichter, L.C. (2013). The microglial activation state regulates migration and roles of matrix-dissolving enzymes for invasion. *J Neuroinflammation* 10**,** 75. doi: 10.1186/1742-2094-10-75

Lopes, K.O., Sparks, D.L., and Streit, W.J. (2008). Microglial dystrophy in the aged and Alzheimer's disease brain is associated with ferritin immunoreactivity. *Glia* 56**,** 1048-1060. doi: 10.1002/glia.20678

Lu, C., Huang, X., Zhang, X., Roensch, K., Cao, Q., Nakayama, K.I., Blazar, B.R., Zeng, Y., and Zhou, X. (2011). miR-221 and miR-155 regulate human dendritic cell development, apoptosis, and IL-12 production through targeting of p27kip1, KPC1, and SOCS-1. *Blood* 117**,** 4293-4303. doi: 10.1182/blood-2010-12-322503

Lu, H.H., Chen, L.K., Cheng, C.Y., Hung, S.L., Lin, S.C., and Chang, K.W. (2009). Areca nut extract-treated gingival fibroblasts modulate the invasiveness of polymorphonuclear leukocytes via the production of MMP-2. *J Oral Pathol Med* 38**,** 79-86. doi: 10.1111/j.1600-0714.2008.00717.x

Lucin, K.M., O'brien, C.E., Bieri, G., Czirr, E., Mosher, K.I., Abbey, R.J., Mastroeni, D.F., Rogers, J., Spencer, B., Masliah, E., and Wyss-Coray, T. (2013). Microglial beclin 1 regulates retromer trafficking and phagocytosis and is impaired in Alzheimer's disease. *Neuron* 79**,** 873-886. doi: 10.1016/j.neuron.2013.06.046

Luo, X.G., Ding, J.Q., and Chen, S.D. (2010). Microglia in the aging brain: relevance to neurodegeneration. *Mol Neurodegener* 5**,** 12. doi: 10.1186/1750-1326-5-12

1750-1326-5-12 [pii].

Lyons, A., Downer, E.J., Crotty, S., Nolan, Y.M., Mills, K.H., and Lynch, M.A. (2007). CD200 ligand receptor interaction modulates microglial activation in vivo and in vitro: a role for IL-4. *J Neurosci* 27**,** 8309-8313. doi: 10.1523/JNEUROSCI.1781-07.2007

Ma, Q., Qiang, J., Gu, P., Wang, Y., Geng, Y., and Wang, M. (2011). Age-related autophagy alterations in the brain of senescence accelerated mouse prone 8 (SAMP8) mice. *Exp Gerontol* 46**,** 533-541. doi: 10.1016/j.exger.2011.02.006

Ma, W., Cojocaru, R., Gotoh, N., Gieser, L., Villasmil, R., Cogliati, T., Swaroop, A., and Wong, W.T. (2013). Gene expression changes in aging retinal microglia:

relationship to microglial support functions and regulation of activation. *Neurobiol Aging* 34**,** 2310-2321. doi: 10.1016/j.neurobiolaging.2013.03.022

Maezawa, I., Zimin, P.I., Wulff, H., and Jin, L.W. (2011). Amyloid-beta protein oligomer at low nanomolar concentrations activates microglia and induces microglial neurotoxicity. *J Biol Chem* 286**,** 3693-3706. doi: 10.1074/jbc.M110.135244

Malaquin, N., Vercamer, C., Bouali, F., Martien, S., Deruy, E., Wernert, N., Chwastyniak, M., Pinet, F., Abbadie, C., and Pourtier, A. (2013). Senescent fibroblasts enhance early skin carcinogenic events via a paracrine MMP-PAR-1 axis. *PLoS One* 8**,** e63607. doi: 10.1371/journal.pone.0063607

Martin, B.K., Szekely, C., Brandt, J., Piantadosi, S., Breitner, J.C., Craft, S., Evans, D., Green, R., and Mullan, M. (2008). Cognitive function over time in the Alzheimer's Disease Anti-inflammatory Prevention Trial (ADAPT): results of a randomized, controlled trial of naproxen and celecoxib. *Arch Neurol* 65**,** 896-905. doi: 10.1001/archneur.2008.65.7.nct70006

Mccarthy, K.D., and De Vellis, J. (1980). Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue. *J Cell Biol* 85**,** 890-902. Doi: 10.1083/jcb.85.3.890

Mccray, B.A., and Taylor, J.P. (2008). The role of autophagy in age-related neurodegeneration. *Neurosignals* 16**,** 75-84. doi: 10.1159/000109761

Miller, A.M., and Stella, N. (2009). Microglial cell migration stimulated by ATP and C5a involve distinct molecular mechanisms: quantification of migration by a novel near-infrared method. *Glia* 57**,** 875-883. doi: 10.1002/glia.20813

Minkeviciene, R., Ihalainen, J., Malm, T., Matilainen, O., Keksa-Goldsteine, V., Goldsteins, G., Iivonen, H., Leguit, N., Glennon, J., Koistinaho, J., Banerjee, P., and Tanila, H. (2008). Age-related decrease in stimulated glutamate release and vesicular glutamate transporters in APP/PS1 transgenic and wild-type mice. *J Neurochem* 105**,** 584-594. doi: 10.1111/j.1471-4159.2007.05147.x

Mosher, K.I., and Wyss-Coray, T. (2014). Microglial dysfunction in brain aging and Alzheimer's disease. *Biochem Pharmacol* 88**,** 594-604. doi: 10.1016/j.bcp.2014.01.008

Moussaud, S., and Draheim, H.J. (2010). A new method to isolate microglia from adult mice and culture them for an extended period of time. *J Neurosci Methods* 187**,** 243-253. doi: 10.1016/j.jneumeth.2010.01.017

Napoli, I., and Neumann, H. (2009). Microglial clearance function in health and disease. *Neuroscience* 158**,** 1030-1038. doi: 10.1016/j.neuroscience.2008.06.046

Ni, M., and Aschner, M. (2010). Neonatal rat primary microglia: isolation, culturing, and selected applications. *Curr Protoc Toxicol* Chapter 12**,** Unit 12 17. doi: 10.1002/0471140856.tx1217s43

Nikodemova, M., Small, A.L., Smith, S.M., Mitchell, G.S., and Watters, J.J. (2013). Spinal but not cortical microglia acquire an atypical phenotype with high VEGF, galectin-3 and osteopontin, and blunted inflammatory responses in ALS rats. *Neurobiol Dis*. doi: 10.1016/j.nbd.2013.11.009 [Epub ahed of print].

Nimmerjahn, A., Kirchhoff, F., and Helmchen, F. (2005). Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. *Science* 308**,** 1314-1318. doi: 10.1126/science.1110647

Njie, E.G., Boelen, E., Stassen, F.R., Steinbusch, H.W., Borchelt, D.R., and Streit, W.J. (2012). Ex vivo cultures of microglia from young and aged rodent brain reveal age-related changes in microglial function. *Neurobiol Aging* 33**,** 195 e191-112. doi: 10.1016/j.neurobiolaging.2010.05.008

Noda, M., Nakanishi, H., and Akaike, N. (1999). Glutamate release from microglia via glutamate transporter is enhanced by amyloid-beta peptide. *Neuroscience* 92**,** 1465-1474. doi: 10.1016/S0306-4522(99)00036-6

Norden, D.M., and Godbout, J.P. (2013). Review: microglia of the aged brain: primed to be activated and resistant to regulation. *Neuropathology and applied neurobiology* 39**,** 19-34. doi: 10.1111/j.1365-2990.2012.01306.x

Noren Hooten, N., Abdelmohsen, K., Gorospe, M., Ejiogu, N., Zonderman, A.B., and Evans, M.K. (2010). microRNA expression patterns reveal differential expression of target genes with age. *PLoS One* 5**,** e10724. doi: 10.1371/journal.pone.0010724

O'Neill, L.A., and Kaltschmidt, C. (1997). NF-kappa B: a crucial transcription factor for glial and neuronal cell function. *Trends Neurosci* 20**,** 252-258. doi: 10.1016/S0166-2236(96)01035-1

Ohsawa, K., Irino, Y., Nakamura, Y., Akazawa, C., Inoue, K., and Kohsaka, S. (2007). Involvement of P2X4 and P2Y12 receptors in ATP-induced microglial chemotaxis. *Glia* 55**,** 604-616. doi: 10.1002/glia.20489

Olivieri, F., Rippo, M.R., Procopio, A.D., and Fazioli, F. (2013). Circulating inflamma-miRs in aging and age-related diseases. *Front Genet* 4**,** 121. doi: 10.3389/fgene.2013.00121

Paczek, L., Michalska, W., and Bartlomiejczyk, I. (2008). Trypsin, elastase, plasmin and MMP-9 activity in the serum during the human ageing process. *Age Ageing* 37**,** 318-323. doi: 10.1093/ageing/afn039

Ponomarev, E.D., Veremeyko, T., Barteneva, N., Krichevsky, A.M., and Weiner, H.L. (2011). MicroRNA-124 promotes microglia quiescence and suppresses EAE by deactivating macrophages via the C/EBP-alpha-PU.1 pathway. *Nature Medicine* 17**,** 64-U234. doi: 10.1038/Nm.2266

Ponomarev, E.D., Veremeyko, T., and Weiner, H.L. (2013). MicroRNAs are universal regulators of differentiation, activation, and polarization of microglia and macrophages in normal and diseased CNS. *Glia* 61**,** 91-103. doi: 10.1002/glia.22363

Prinz, M., and Priller, J. (2014). Microglia and brain macrophages in the molecular age: from origin to neuropsychiatric disease. *Nat Rev Neurosci* 15**,** 300-312. doi: 10.1038/nrn3722

Rovina, N., Koutsoukou, A., and Koulouris, N.G. (2013). Inflammation and immune response in COPD: where do we stand? *Mediators Inflamm* 2013**,** 413735. doi: 10.1155/2013/413735

Sailasuta, N., Ernst, T., and Chang, L. (2008). Regional variations and the effects of age and gender on glutamate concentrations in the human brain. *Magn Reson Imaging* 26**,** 667-675. doi: 10.1016/j.mri.2007.06.007

Saura, J., Tusell, J.M., and Serratosa, J. (2003). High-yield isolation of murine microglia by mild trypsinization. *Glia* 44**,** 183-189. doi: 10.1002/glia.10274

Schmid, C.D., Melchior, B., Masek, K., Puntambekar, S.S., Danielson, P.E., Lo, D.D., Sutcliffe, J.G., and Carson, M.J. (2009). Differential gene expression in LPS/IFNgamma activated microglia and macrophages: in vitro versus in vivo. *J Neurochem* 109 Suppl 1**,** 117-125. doi: 10.1111/j.1471-4159.2009.05984

Schwartz, M., Butovsky, O., Bruck, W., and Hanisch, U.K. (2006). Microglial phenotype: is the commitment reversible? *Trends Neurosci* 29**,** 68-74. doi: 10.1016/j.tins.2005.12.005

Severino, J., Allen, R.G., Balin, S., Balin, A., and Cristofalo, V.J. (2000). Is beta-galactosidase staining a marker of senescence in vitro and in vivo? *Exp Cell Res* 257**,** 162-171. doi: 10.1006/excr.2000.4875

Sikora, E., Arendt, T., Bennett, M., and Narita, M. (2011). Impact of cellular senescence signature on ageing research. *Ageing Res Rev* 10**,** 146-152. doi: 10.1016/j.arr.2010.10.002

Silva, S.L., Vaz, A.R., Barateiro, A., Falcão, A.S., Fernandes, A., Brito, M.A., Silva, R.F., and Brites, D. (2010). Features of bilirubin-induced reactive microglia: from phagocytosis to inflammation. *Neurobiol Dis* 40**,** 663-675. doi: 10.1016/j.nbd.2010.08.010

Silva, S.L., Vaz, A.R., Diogenes, M.J., Van Rooijen, N., Sebastiao, A.M., Fernandes, A., Silva, R.F., and Brites, D. (2012). Neuritic growth impairment and cell death by unconjugated bilirubin is mediated by NO and glutamate, modulated by microglia,

and prevented by glycoursodeoxycholic acid and interleukin-10. *Neuropharmacology*. doi: 10.1016/j.neuropharm.2012.02.002

Simpson, J.L., Mcdonald, V.M., Baines, K.J., Oreo, K.M., Wang, F., Hansbro, P.M., and Gibson, P.G. (2013). Influence of age, past smoking, and disease severity on TLR2, neutrophilic inflammation, and MMP-9 levels in COPD. *Mediators Inflamm* 2013**,** 462934. doi: 10.1155/2013/462934

Sinha, M., Ghose, J., and Bhattarcharyya, N.P. (2011). Micro RNA -214,-150,-146a and-125b target Huntingtin gene. *RNA Biol* 8**,** 1005-1021. doi: 10.4161/rna.8.6.16035

Smith-Vikos, T., and Slack, F.J. (2012). MicroRNAs and their roles in aging. *J Cell Sci* 125**,** 7-17. doi: 10.1242/jcs.099200

Solito, E., and Sastre, M. (2012). Microglia function in Alzheimer's disease. *Front Pharmacol* 3**,** 14. doi: 10.3389/fphar.2012.00014

Streit, W.J., Braak, H., Xue, Q.S., and Bechmann, I. (2009). Dystrophic (senescent) rather than activated microglial cells are associated with tau pathology and likely precede neurodegeneration in Alzheimer's disease. *Acta Neuropathol* 118**,** 475-485. doi: 10.1007/s00401-009-0556-6

Streit, W.J., Sammons, N.W., Kuhns, A.J., and Sparks, D.L. (2004). Dystrophic microglia in the aging human brain. *Glia* 45**,** 208-212. doi: 10.1002/glia.10319

Streit, W.J., and Xue, Q.S. (2012). Alzheimer's disease, neuroprotection, and CNS immunosenescence. *Front Pharmacol* 3**,** 138. doi: 10.3389/fphar.2012.00138

Streit, W.J., and Xue, Q.S. (2013). Microglial senescence. *CNS Neurol Disord Drug Targets* 12**,** 763-767. doi: 10.2174/18715273113126660176

Szabo, M., and Gulya, K. (2013). Development of the microglial phenotype in culture. *Neuroscience* 241**,** 280-295. doi: 10.1016/j.neuroscience.2013.03.033

Taganov, K.D., Boldin, M.P., Chang, K.J., and Baltimore, D. (2006). NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. *Proc Natl Acad Sci U S A* 103**,** 12481-12486. doi: 10.1073/pnas.0605298103

Takaki, J., Fujimori, K., Miura, M., Suzuki, T., Sekino, Y., and Sato, K. (2012). L-glutamate released from activated microglia downregulates astrocytic L-glutamate transporter expression in neuroinflammation: the 'collusion' hypothesis for increased extracellular L-glutamate concentration in neuroinflammation. *J Neuroinflammation* 9**,** 275. doi: 10.1186/1742-2094-9-275

Tanaka, J., Toku, K., Sakanaka, M., and Maeda, N. (1999). Morphological differentiation of microglial cells in culture: involvement of insoluble factors derived from astrocytes. *Neurosci Res* 34**,** 207-215. doi: 10.1016/S0168-0102(99)00041-3

Torres-Platas, S.G., Comeau, S., Rachalski, A., Bo, G.D., Cruceanu, C., Turecki, G., Giros, B., and Mechawar, N. (2014). Morphometric characterization of microglial phenotypes in human cerebral cortex. *J Neuroinflammation* 11**,** 12. doi: 10.1186/1742-2094-11-12

Varnum, M.M., and Ikezu, T. (2012). The classification of microglial activation phenotypes on neurodegeneration and regeneration in Alzheimer's disease brain. *Arch Immunol Ther Exp (Warsz)* 60**,** 251-266. doi: 10.1007/s00005-012-0181-2

Von Bernhardi, R., Tichauer, J., and Eugenin-Von Bernhardi, L. (2011). Proliferating culture of aged microglia for the study of neurodegenerative diseases. *J Neurosci Methods* 202**,** 65-69. doi: 10.1016/j.jneumeth.2011.08.027

Weggen, S., Eriksen, J.L., Das, P., Sagi, S.A., Wang, R., Pietrzik, C.U., Findlay, K.A., Smith, T.E., Murphy, M.P., Bulter, T., Kang, D.E., Marquez-Sterling, N., Golde, T.E., and Koo, E.H. (2001). A subset of NSAIDs lower amyloidogenic Abeta42 independently of cyclooxygenase activity. *Nature* 414**,** 212-216. doi: 10.1038/35102591

Williamson, L.L., Sholar, P.W., Mistry, R.S., Smith, S.H., and Bilbo, S.D. (2011). Microglia and memory: modulation by early-life infection. *J Neurosci* 31**,** 15511-15521. doi: 10.1523/JNEUROSCI.3688-11.2011

Wojtera, M., Sobow, T., Kloszewska, I., Liberski, P.P., Brown, D.R., and Sikorska, B. (2012). Expression of immunohistochemical markers on microglia in Creutzfeldt-Jakob disease and Alzheimer's disease: morphometric study and review of the literature. *Folia Neuropathol* 50**,** 74-84.

Wong, W.T. (2013). Microglial aging in the healthy CNS: phenotypes, drivers, and rejuvenation. *Front Cell Neurosci* 7**,** 22. doi: 10.3389/fncel.2013.00022

Yao, L., Kan, E.M., Lu, J., Hao, A., Dheen, S.T., Kaur, C., and Ling, E.A. (2013). Toll-like receptor 4 mediates microglial activation and production of inflammatory mediators in neonatal rat brain following hypoxia: role of TLR4 in hypoxic microglia. *J Neuroinflammation* 10**,** 23. doi: 10.1186/1742-2094-10-23

Yu, H.M., Zhao, Y.M., Luo, X.G., Feng, Y., Ren, Y., Shang, H., He, Z.Y., Luo, X.M., Chen, S.D., and Wang, X.Y. (2012). Repeated lipopolysaccharide stimulation induces cellular senescence in BV2 cells. *Neuroimmunomodulation* 19**,** 131-136. doi: 10.1159/000330254

Zhang, G., Li, J., Purkayastha, S., Tang, Y., Zhang, H., Yin, Y., Li, B., Liu, G., and Cai, D. (2013). Hypothalamic programming of systemic ageing involving IKK-beta, NF-kappaB and GnRH. *Nature* 497**,** 211-216. doi: 10.1038/nature12143

Chapter 3

KEY AGEING-ASSOCIATED ALTERATIONS IN PRIMARY MICROGLIA RESPONSE TO BETA-AMYLOID STIMULATION

Cláudia Caldeira[1], Carolina Cunha[1], Ana Rita Vaz[1,2], Ana Sofia Falcão[1,2], Andreia Barateiro[1], Elsa Seixas[3], Adelaide Fernandes[1,2], Dora Brites[1,2]

[1] Research Institute for Medicines (iMed.ULisboa), Faculdade de Farmácia, Universidade de Lisboa, Lisbon, Portugal

[2] Department of Biochemistry and Human Biology, Faculdade de Farmácia, Universidade de Lisboa, Lisbon, Portugal

[3] Obesity LAboratory, Instituto Gulbenkian de Ciência, 2780-156 Oeiras, Portugal

Front Aging Neurosc. **(2017) (submitted)**

Acknowledgments

This work was supported by FEDER (COMPETE Programme) and by National funds (FCT - Fundação para a Ciência e a Tecnologia) Project FCT-PEst-OE/SAU/UI4013 to iMed.ULisboa and Project FCT- EXPL/NEU-NMC/1003/2013 to AF, and, in part, by the EU Joint Programme - Neurodegenerative Disease Research (JPND) MADGIC project JPco-fuND/0003/2015 to DB, as well as by the prize Edgar Cruz e Silva 2012 from Grupo de Estudo do Envelhecimento Cerebral e Demência to DB. ASF holds a post-doctoral research position (C2007-FFUL/UBMBE/02/2011), and ARV together with AB postdoctoral research fellowships (SFRH/BPD/76590/2011 and SFRH/BPD/96794/2013, respectively), and C Cunha is recipient of a PhD fellowship (SFRH/BD/91316/2012), all from FCT. The funding organization had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Abstract

Alzheimer's disease (AD) is characterized by a progressive cognitive decline and self-aggregation of amyloid-β peptide (Aβ) into oligomers and amyloid fibrils that accumulate as senile plaques. Neuroinflammation is considered a hallmark of AD pathogenesis and is associated with the release of inflammatory mediators by the reactive astrocytes and activated microglia adjacent to Aβ plaques. To note, however, that microglia may exert either neuroprotective or neurotoxic responses depending on the physiological conditions they encounter and the phenotype they assume. In this study, we set out to determine if the 2 days *in vitro* (DIV) activated microglia in the course of the isolation procedure and the 16 DIV aged cultured microglia react differently to Aβ stressful stimulus. Cultured microglia were treated with 1000 nM $A\beta_{1-42}$ for 24 h, which showed to not cause cell death, but to induce morphometric alterations that included soma volume increase and process shortening, less evident in 16 DIV microglia. Here we observed that several neuroprotective functions, namely phagocytosis and migration abilities, as well as autophagy, were impaired in *in vitro* aged microglia. Interestingly, Aβ treatment reduced the phagocytic capacity of the activated 2 DIV microglia and promoted autophagy, while increasing the activity of the senescence-associated β-galactosidase and the expression of miR-146a. Age-dependent changes included the decrease in the expression of inflammatory mediators and surface receptors from "young" to aged cells upon treatment with $A\beta_{1-42}$. Additionally, Aβ caused a downregulation of miR-155 and miR-124, and reduced the CD11b+ subpopulation in 2 DIV microglia. Intriguingly, it increased the number of CD86+ cells in aged microglia. Finally, we observed that mixed M1 and M2 subpopulations occur after Aβ treatment, but that responses are largely attenuated in the aged microglia. Our data show the existence of key-aging associated alterations, with a predominant Aβ-induced microglia M1 polarization and loss of phagocytic ability in the 2 DIV cells, together with a decreased reactivity towards Aβ in the 16 DIV microglia. These findings help in improving our understanding on the heterogeneous responses that microglia may have along the progression of AD disease and imply that therapeutic approaches may differ from early to late stages.

Keywords: Alzheimer's Disease, amyloid-β peptide; neuroinflammation; aged-cultures microglia; inflammatory-microRNAs; M1/M2 microglia subtypes; CD11b; CD86

1. Introduction

Alzheimer's disease (AD) is the most common dementing disorder in the elderly affecting around 35.6 million people worldwide and is expected to duplicate in the years to come (Prince et al., 2013). Accumulation of extracellular β-amyloid (Aβ) plaques within the brain is suggested to result from the cleavage of the amyloid precursor protein (APP) which quantity is elevated in AD (Rubio-Perez and Morillas-Ruiz, 2012). Deposition of Aβ was shown to trigger the activation of both astrocytes and microglia leading to the production of pro-inflammatory cytokines such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α, among other compounds (Wyss-Coray, 2006), triggering neuroinflammation that is known to contribute to AD progression and severity (Heneka et al., 2015a). However, treatment with nonsteroidal anti-inflammatory drugs (NSAIDs) has consistently failed in AD patients (von Bernhardi, 2010), unless administrated early in the disease (Varvel et al., 2009). Recent studies in rodent models have shown that fenamate NSAIDS decrease microglia activation and IL-1β processing, which led the Authors to suggest that they may be repurposed as inhibitors of the NLRP3 inflammasome (Daniels et al., 2016). If microglial malfunction trigger AD pathogenesis, contribute to altered microenvironment, and precede or facilitate AD onset (Regen et al., 2017), clarification of the underlying molecular mechanisms should be clarified.

Microglia are the first line and the main immune defense against disease and injury in the central nervous system (CNS). When activated by stress stimuli the cells migrate and restrict the damage by surrounding the site lesion and phagocytizing cell debris. Microglia are activated by oligomeric and fibrillar species of Aβ, as well as by molecules derived from degenerated neurons (Mizuno, 2012) and have shown capacity in amyloid removal (Morgan, 2009). However, at least in some conditions, the phagocytic and inflammatory phenotypes may alternate along time, depending on the microglia-induced polarization subtype (Silva et al., 2010). Actually, and depending on the stimuli, microglia may comprise a family of cells with diverse phenotypes exerting beneficial or destructive effects (Schwartz et al., 2006). Current researches also propose that age-dependent neuroinflammatory changes may play a significant role in the decreased neurogenesis and cognitive impairments observed in AD (Lynch et al., 2010; Varnum and Ikezu, 2012). In addition, it has been suggested that these cells may lose their ability to phagocytose Aβ with age and disease progression and that in late disease stages inflammation no longer exists (Floden and Combs, 2011; Wojtera et al., 2012). As so, there is an urgent need to understand age-dependent changes in microglia function and response to stimuli to better recognize the role of microglia in early and late-stages of Alzheimer's disease.

Over the years there have been studies intended to identify different activation phenotypes in several *in vitro* and *in vivo* models, as well as in AD brain specimens,

trying to fit them into current polarization schemes (Walker and Lue, 2015). Although microglia priming and M1-like activation state in AD is suggested in the most of the works (Heneka et al., 2015b; Hoeijmakers et al., 2016), increased expression of Arginase 1 (Colton et al., 2006) and co-expression of M1, M2a, M2b and M2c markers were also detected (Wilcock, 2012; Sudduth et al., 2013). In addition, later studies identified five ramified, hypertrophic, dystrophic, rod-shaped, and amoeboid microglia morphological phenotypes in AD patient autopsied samples, with increased prevalence of dystrophic microglia in cases of dementia with Lewy bodies (Bachstetter et al., 2015). Disparate results obtained so far derive from the difficulties in selecting experimental models that better recapitulate the *in vivo* AD condition. For instance, *in vitro* microglial models, such as microglial cell lines, or primary microglia isolated from embryonic (Gingras et al., 2007) or neo-natal animals (Floden and Combs, 2007), though largely used (Moussaud and Draheim, 2010), do not mimic the adult behavior cells (Sierra et al., 2007). Furthermore, it was demonstrated that primary cultures of microglial cells change their activation profile over time in culture (Cristóvão et al., 2010), thus contributing to data inconsistency.

Since AD is considered an age-related disease, the use of aging animal models has been proposed to circumvent the difficulties arising from researching the nervous system aging (Bachstetter et al., 2015). However a lot of problems should be considered. Actually, the need of waiting for 2-3 years for animals to age to assess differences in cell function and with only the survivors being used, together with the variability in the results that were obtained (Birch et al., 2014), have contributed to the failure in understanding many of the elderly processes and in successful innovative strategic approaches to AD. Therefore, our aged *in vitro* culture microglia model (Caldeira et al., 2014) may add additional information on the controversial microglia phenotypes in AD onset and progression, while allowing the work with aged microglia, once there are no processes to isolate degenerating microglia for experimentation (Njie et al., 2012).

In the present study we assumed that the recently isolated microglia maintained for 2 days *in vitro* (DIV) and the 16 DIV aged cultured microglia represent different cell populations that may react differently to the Aβ stress stimulus. These subtypes may underlie diverse vulnerabilities along AD progression from onset to late stages and may serve as models to better comprehending cell malfunction changes deriving from the accumulation of Aβ and age-related alterations, not clarified till now. Based in our previous data (Caldeira et al., 2014), we hypothesized that the 2 DIV microglia phenotype mainly resembled the activation of the cell in the subacute inflammation state, while the 16 DIV cells more closely represented the inability of the cell to mount an efficient response against stressor stimuli. Hence, our main interest was to examine the behavior

of these *in vitro* cultured microglia phenotypes, young/reactive (2 DIV) and aged/desensitized (16 DIV) cells, when facing a non-toxic mixture of $A\beta_{1-42}$ oligomers and fibrils species at a concentration of 1000 nM. For that, we assessed cell morphology, phagocytic ability, migration capacity, autophagy and senescence markers, as well as the inflammatory-associated microRNAs, mediators, key regulatory receptors and inflammasome, together with MMP2 and MMP9 activation. Further, we evaluated gene expression of microglia phenotype M1 and M2 biomarkers and explored their subtype distribution.

Our results indicate that $A\beta_{1-42}$, although prompting an acute inflammatory reaction, promote the switch of the activated microglia towards heterogeneous populations, while eliciting microglia senescence and affecting phagocytic function in the 2 DIV *in vitro* microglia. Data also highlight the presence of an increased population of CD86+ microglia in the 16 DIV cells, which revealed decreased ability to mount cellular immune responses and compromised neuroprotective effects when exposed to $A\beta_{1-42}$, as compared to 2 DIV cells. Altogether, we may hypothesize that different microglia polarized cells are suggestive to distinctly contribute to AD initiation and progression, thus requiring therapeutic diversity.

2. Materials and Methods

2.1. Animals

Animal care followed the recommendations of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Council Directive 86/609/EEC) and National Law 1005/92 (rules for protection of experimental animals). All animal procedures were approved by the Institutional animal care and use committee. Every effort was made to minimize the number of animals used and their suffering.

2.2. Primary culture of microglia

Mixed glial cultures were prepared from 1-to-2 day-old CD1 mice, as previously described (McCarthy and de Vellis, 1980), with minor modifications (Gordo et al., 2006). Cells (4×10^5 cells/cm^2) were plated on uncoated 12-well tissue culture plates (with 18 mm coverslips) or 75-cm^2 culture flasks in culture medium (DMEM-Ham's F12 medium supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, nonessential amino acids 1X, 10% fetal bovine serum (FBS), and 1% antibiotic-antimycotic solution) and maintained at 37ºC in a humidified atmosphere of 5% CO_2.

Microglia were isolated as previously described (Saura et al., 2003). Briefly, after 21 days in culture, microglia were obtained by mild trypsinization with a trypsin-EDTA solution diluted 1:3 in DMEM-Ham's F12 for 45 to 60 min. The trypsinization resulted in detachment of an upper layer of cells containing all the astrocytes, whereas the microglia remained attached to the bottom of the well. The medium containing detached cells was removed and the initial mixed glial-conditioned medium was added. The use of 21 days *in vitro* (DIV) cells intends to achieve the maximal yield and purity of the cultures. In fact, astrocyte contamination was less than 2%, as assessed by immunocytochemical staining with a primary antibody against GFAP followed by a species-specific fluorescent-labeled secondary antibody. Neuronal contamination was 0%, as assessed by immunocytochemical staining with a primary antibody against MAP-2 followed by a species-specific fluorescent-labeled secondary antibody (Silva et al., 2010).

2.3. Treatment of microglia with a mixture of $A\beta_{1-42}$ oligomers and fibrils

$A\beta_{1-42}$ peptide was diluted in DMEM-Ham's F12 culture medium to a stock concentration of 111 µM and allowed to incubate for 24 h at 37°C to preaggregate the peptides, as formerly indicated (Hjorth et al., 2013). In previous studies (Falcão et al., 2016), we verified that our $A\beta_{1-42}$ solution were mainly constituted by large oligomers and fibrils with a small proportion of monomers and dimers, after a 24 h period of time, as we used in the incubation assays.

Isolated microglia were differently aged in culture for 2 and 16 DIV to have two diverse microglia subtypes in accordance with our prior publication (Caldeira et al., 2014). The 2 DIV cells to represent activated microglia that follows the acute process of isolation, and the 16 DIV to mimic an aged and less responsive population. Both *in vitro* aged cells were incubated with 50 nM and 1000 nM $A\beta_{1-42}$, during 24 h, at 37ºC, although the lower concentration was later abandoned in favor of the more consistent results obtained with the higher one. Cells incubated in the absence of $A\beta_{1-42}$ were used as controls.

2.4. Determination of cell death

To evaluate microglia cell death we used phycoerythrin-conjugated annexin V (V-PE) and 7-amino-actinomycin D (7-AAD) (Guava Nexin® Reagent, #4500-0450, Millipore) to determine the percentage of viable, early-apoptotic and late-apoptotic/necrotic cells by flow cytometry. After incubation, microglia were trypsinized, added to the cells present in the incubation media and then stained with annexin V-PE and 7-AAD, following manufacturer's instructions, and analyzed on a Guava easyCyte

5HT flow cytometer (Guava Nexin® Software module, Millipore), as previously described (Barateiro et al., 2012). The three populations of cells that can be distinguished by this assay are the viable cells (annexin V-PE and 7-AAD negative), the early apoptotic cells (annexin V-PE positive and 7-AAD negative), and the cells in late stages of apoptosis or dead cells (annexin V-PE and 7-AAD positive).

2.5. Cell morphological analysis

For morphological analysis, cells were fixed for 20 min with freshly prepared 4% (w/v) paraformaldehyde in phosphate buffer saline (PBS). Standard immunocytochemical technique used a primary antibody raised against Iba-1 (rabbit, 1:250; Wako Pure Chemical Industries Ltd, Osaka, Japan), and a secondary Alexa Fluor 594 goat anti-rabbit (1:1000; Invitrogen Corporation, Carlsbad, CA, USA). To identify the total number of cells, microglial nuclei were stained with Hoechst 33258 dye. Fluorescence was visualized using an AxioCam HRm camera adapted to an AxioSkope® microscope (Zeiss). Pairs of U.V. and red-fluorescence images of ten random microscopic fields (original magnification: 400X) were acquired per sample. To better characterize microglia morphology we used the particle measurement feature in ImageJ (1.47v, USA) to automatically obtain the 2D area, perimeter, circularity, and Feret's diameter of single microglia cells. Circularity of microglia was calculated using the formula: Circularity = 4π (area/perimeter^2). A circularity value of 1.0 indicates a perfectly circular cell, and values near zero indicate elongated and ramified cells. Feret's (maximum) diameter, a measure of cell length, is the greatest distance between any two points along the cell perimeter.

2.6. Microglia migration assessment

Cell migration is often assessed with the classic Boyden Chamber assay, where cells loaded in the upper well are allowed to migrate through filter pores to the lower well of the chamber. Assays were performed in a 48-well microchemotaxis Boyden chamber (Neuro Probe, Gaithersburg, MD, USA), as previously described (Miller and Stella, 2009), with minor modifications. The bottom wells were filled with control medium (DMEM-Ham's F12) and Aβ (1000 nM) to evaluate the ability of microglia to move towards Aβ. ATP (10 μM) applied in the lower well was also used as a positive control for microglia migration, since it is a known chemoattractant for microglia. The 8 μm diameter polycarbonate membranes with polyvinylpyrrolidone (PVP) surface treatment was equilibrated in control medium and after chamber set up, 50 μl of cell suspension containing 2 x 10^4 was added to each top well. After 6 h incubation in a CO_2 incubator at 37ºC to allow microglia migration, membrane was fixed with cold methanol and cells

stained with 10% Giemsa in PBS. Non-migrated cells on the upper side of the membrane were wiped off with a filter wiper. The rate of migration was determined by counting the cells on the lower membrane surface, using 10 microscopic fields (original magnification: 100X) that cover all the well. Images were acquired with a Leica DFC490 camera adapted to an AxioSkope HBO50 microscope. For each experiment, at least data from three wells per condition were acquired.

2.7. Evaluation of metalloproteinase-2 and -9 activities

Assessment of matrix metalloproteinases (MMPs)-2 and MMP9 activities in the extracellular medium was based on their ability to degrade gelatin. For that, 20 µl of incubation medium was resolved using a SDS-PAGE zymography of 0.1% gelatin – 10% acrylamide gel. After electrophoresis, gels were washed for 1 h with 2.5% TritonX-100 (in 50 nM $CaCl_2$; 1 µM $ZnCl_2$) to remove SDS and renature MMP species in the gel. To allow gelatin degradation by MMPs, gels were incubated overnight, at 37ºC, in the developing buffer (50 mM Tris pH 7.4; 5 mM $CaCl_2$; 1 µM $ZnCl_2$). For enzyme activity analysis, the gels were stained with 0.5% Coomassie Brilliant Blue R-250 and distained in 30% ethanol/10% acetic acid/H_2O. Gelatinase activity, detected as a white band on a blue background, was measured by computerized image analysis (Image Lab™ Software 3.0, Bio-Rad Laboratories Inc., USA) and normalized to cellular protein content (Silva et al., 2010).

2.8. Gene expression profiling

Quantitative RealTime PCR (qRT-PCR) was performed for mRNA expression, as usual in our laboratory (Barateiro et al., 2013). Total RNA was extracted from microglia using TRIzol® (LifeTechnologies), according to manufacturer's instructions. Total RNA was quantified using Nanodrop ND-100 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Aliquots of 0.5 µg of total RNA were treated with DNase I and then reverse transcribed cDNA using oligo-dT primers and SuperScript II Reverse Transcriptase under the recommended conditions. qRT-PCR was performed on a 7300 Real time PCR System (Applied Biosystems) using a SYBR Green qPCR Master Mix (Fermentas) and β-actin as an endogenous control to normalize the expression level of the different genes. Primer sequences that were used are indicated in **Table 3.1**. PCR was performed in 96 well plates and triplicate analysis was accomplished for each sample. No-template control was included for each amplificate. Cycling conditions were 94ºC for 3 min followed by 40 cycles at 94ºC for 0.15 min, 62ºC for 0.2 min and 72ºC for 0.15 min. A melt-curve analysis was used to verify the specificity of the amplification,

immediately after the amplification protocol. Non-specific products of PCR were not found in any case. Relative mRNA concentrations were calculated using the Pfaffl modification of the ΔΔCT equation (CT, cycle number at which fluorescence passes the threshold level of detection), taking into account the efficiencies of individual genes. The results were normalized to β-actin in the same sample and the initial amount of the template of each sample determined as relative expression by the formula 2-ΔΔCT. ΔCT in each sample derives from the difference between the mean CT value of each gene and the mean CT value of β-actin. ΔΔCT of one sample is the difference between its ΔCT value and ΔCT of the selected reference, in our case the 2 DIV control cells.

2.9. Determination of microglia senescence and autophagy

Microglial senescence was determined using the Cellular senescence assay kit (Millipore) that evaluates the activity of senescence-associated β-galactosidase, according to the manufacturer instructions. Microglial nuclei were counterstained with hematoxylin. Light microscopy images of ten random microscopic fields (original magnification: 400X) were acquired per sample. Turquoise blue stained microglia were considered senescent and their percentage calculated relatively to the total number of cells.

Autophagy was determined by immunocytochemistry based on the punctate pattern of the microtubule-associated-protein-light-chain-3 (LC3). Measurements of Beclin-1 bands by Western Blot were normalized to the correspondent β-actin bands, as previously described (Caldeira et al., 2014).

2.10. Evaluation of microglial phagocytic ability

The efficiency of the microglial phagocytosis was assessed by counting the number of ingested beads per cell, considering the total number of cells, to obtain the average amount of ingested beads per cell, and by the percentage of cells phagocytosing less than 5, 5-10 or more than 10 beads. The method consisted in incubating the primary microglial cultures, differently aged in culture with 0.0025% (w/w) of 1 μm fluorescent latex beads (Sigma Chemical Co., St. Louis, MO, USA) for 75 min at 37ºC. Thereafter, cells were fixed with freshly prepared 4% (w/v) paraformaldehyde in PBS. Microglia were stained for Iba-1 and nuclei counterstained with Hoechst dye. Fluorescence was visualized and acquired as above mentioned.

Table 3. 1 – List of primer sequences used for gene expression

Gene	Sense (5'-3')	Antisense (5'-3')
B-actin	GCTCCGGCATGTGCAA	AGGATCTTCATGAGGTAGT
MFG-E8	TGACTTTGGACACACAGCGT	GTGTAGAACAACGGGAGGCT
TNF-α	TACTGAACTTCGGGGTGATTGGTCC	CAGCCTTGTCCCTTGAAGAGAACC
IL-1β	CAGGCTCCGAGATGAACAAC	GGTGGAGAGCTTTCAGCTCATA
IL-6	CCGGAGAGGAGACTTCACAG	GGAAATTGGGGTAGGAAGGA
HMGB1	CTCAGAGAGGTGGAAGACCATGT	GGGATGTAGGTTTTCATTTCTCTTTC
IL-18	TGGTTCCATGCTTTCTGGACTCCT	TTCCTGGGCCAAGAGGAAGTG
NLRP3	TGCTCTTCACTGCTATCAAGCCCT	ACAAGCCTTTGCTCCAGACCCTAT
TLR2	TGCTTTCCTGCTGAAGATTT	TGTACCGCAACAGCTTCAGG
TLR4	ACCTGGCTGGTTTACACGTC	GTGCCAGAGACATTGCAGAA
CX3CR1	TGCTCTTCACGTTCGGTCTG	CTCAAGGCCAGGTTCAGGAG
iNOS	ACCCACATCTGGCAGAATGAG	AGCCATGACCTTTCGCATTAG
MHC class II	TGGGCACCATCTTCATCATTC	GGTCACCCAGCACACCACTT
Arginase 1	CTTGGCTTGCTTCGGAACTC	GGAGAAGGCGTTTGCTTAGTTC
TGF-β	CAGAGCTGCGCTTGCAGAG	GTCAGCAGCCGGTTACCAAG

2.11. Detection of specific microRNA expression changes

To evaluate changes in microRNAs (miRNAs) with a crucial role in microglia function/dysfunction we assessed the expression of miR-124, miR-155 and miR-146a by qRT-PCR. Total RNA was extracted from primary microglia cultures using the miRCURY™ Isolation Kit – Cells (Exiqon), according to the manufacturer's recommendations for cultured cells. Briefly, after cell lysis, the total RNA was adsorbed to a silica matrix, washed with the recommended buffers and eluted with 35 µl RNase-free water by centrifugation. After RNA quantification, conversion to cDNA was performed using the universal cDNA Synthesis Kit (Exiqon) and 20 ng total RNA according to the following protocol: 60 min at 42ºC followed by heat-inactivation of the reverse transcriptase for 5 min at 95ºC. qRT-PCR was performed in an 7300 Real time PCR System (Applied Biosystems) using 96-well plates. For miRNA quantification the miRCURY LNA™ Universal RT microRNA PCR system (Exiqon) was used in combination with pre-designed primers (Exiqon), represented in **Table 3.2** using SNORD110 as reference gene. The reaction conditions consisted of polymerase activation/denaturation and well-factor determination at 95ºC for 10 min, followed by 50

amplification cycles at 95ºC for 10 s and 60ºC for 1 min (ramp-rate 1.6º/s). The miRNA fold increase or fold decrease with respect to control samples was determined by the Pfaffl method, taking into consideration different amplification efficiencies of miRNAs in all experiments. The amplification efficiency for each target was determined according to the formula: $E = 10^{(-1/S)} - 1$, where S is the slope of the obtained standard curve.

Table 3. 2 - List of primer sequences used for microRNA expression

miRNA	Target Sequence (5'-3')
mmu-miR-155-5p	UUAAUGCUAAUUGUGAUAGGGGU
hsa-miR-146a-5p	UGAGAACUGAAUUCCAUGGGUU
hsa-miR-124-3p	UAAGGCACGCGGUGAAUGCC
SNORD110	Reference gene

2.12. Separation of CD11b and CD86 microglia populations by flow cytometry

Cells were resuspended in PBS and kept in flow buffer (PBS plus 2% FBS and 0.02% sodium azide). To prevent non-specific binding, cells were incubated for 20 min with CD16/CD32 (1:100) to block Fc receptors, at 4°C. Afterwards, cell suspension was incubated with the fluorescent labeled antibodies (CD11b PerCp-Cy5, CD45 PE and CD86 Bio-SAV PE) for 30 min, at 4°C (1:100). Following the incubation, cells were washed with flow buffer, incubated with streptavidin (1:100) for the CD86 Bio-SAV PE antibody during 30 min, and then resuspended in flow buffer. Expression of surface antigens was evaluated using the BD FACSCalibur flow cytometer and data analyzed using the FlowJo software.

2.13. Statistical analysis

Results of at least four different experiments are expressed as mean ± SEM. Significant differences between control and Aβ treated groups were determined by *t*-test. To compare the effects of Aβ treatment and microglia differently aged in culture, two-way ANOVA was used using GraphPad Prism® 5.0 (GraphPad Software, San Diego, CA, USA). Statistical significance was considered for $p < 0.05$.

3. Results

3.1. Microglia treated with Aβ do not show age-dependent changes in cell viability

In the present work we used our established model of reactive and age-like microglia phenotypes, in which the cells are maintained for 2 DIV and 16 DIV in culture, respectively, after isolating microglia from the mixed cultures with astrocytes (Caldeira et al., 2014). Our main interest was to see whether activated/young cells would respond differently from the aged cells to Aβ. For that, we decided to incubate the cells with a mixture of Aβ oligomers and fibrils, as we have accomplished in the N9 microglial cell line (Falcão et al., 2016), to recapitulate the neuropathology of AD associated to the different activation processes of microglia by such species (Sondag et al., 2009). Although we have tested 50 nM and 1000 nM Aβ concentrations, already used in our previous study (Falcão et al., 2016), the effects obtained in differently aged microglia function/dysfunction were more notorious for the highest level, reason why we decided to only assess and present the results obtained with Aβ 1000 nM for a 24 h treatment period.

As a first step, and in order to guarantee that the viability of the aged-cultured microglia (16 DIV) was equivalent to that of the acutely (2 DIV) isolated cells, we determined the percentage of viable, early-apoptotic and late-apoptotic/necrotic cells by flow cytometry in both adherent and detached cells, as described in Materials and Methods Section. As indicated in **Table 3.3**, although a slight increase was obtained in the number of cells showing late-apoptosis/necrosis by treatment with Aβ, namely in the 16 DIV microglia, the lack of significance of such effects attests no direct influence on the events that will be presented in the following sections.

Table 3. 3 – Microglia viability is not altered by amyloid-β treatment

	2 days *in vitro*		**16 days *in vitro***	
	Control	**Aβ (1000nM)**	**Control**	**Aβ (1000nM)**
Viable	73.6 (±9.1)	65.6 (±6.7)	70.7 (±7.1)	66.5 (±5.7)
Early-apoptosis	18.8 (±8.6)	17.8 (±4.8)	21.7 (±8.1)	21.6 (±5.3)
Late-apoptosis/ necrosis	2.9 (±1.4)	3.7 (±1.4)	5.8 (±1.0)	9.1 (±2.4)

Results are expressed as percentage of the total number ofcells. Values are means ± SEM from at least four independent experiments. Microglial cells were kept in culture for 2 days *in vitro* (DIV) and 16 DIV and treated with amyloid-β (Aβ) at 1000 nM for 24h. The percentage of viable microglia and microglia in early- and late-apoptosis/necrosis was determined by flow cytometer with phycoerythrin-conjugated annexin V (annexin V-PE) and 7-amino-actinomycin D (7-AAD). The three populations were distinguished as follow: viable cells (annexin V-PE and 7-AAD negative), early apoptotic cells (annexin V-PE positive and 7-AAD negative), and late stages of apoptosis or dead cells (annexin V-PE and 7-AAD positive).

3.2. Young and aged cultured microglia show soma enlargement by Aβ treatment

Characterization of morphometric features of microglial cells has been associated to microglia polarization and state of inflammatory processes (Torres-Platas et al., 2014). While the ramified morphology relates with the surveilling cell type function, the amoeboid microglia are associated to activation and believed to favor phagocytosis and mobility (Lull and Block, 2010).

The cells cultured for 2 DIV (young/reactive) show a predominant amoeboid morphology as a consequence of the acutely isolation protocol that leads to the activation of the cells (**Figure 3.1A**), as we previously stated (Caldeira et al., 2014). When maintained in culture for 16 DIV (aged), the cells exhibit polarized and ramified populations, including rod-like and bipolar morphology appearance, thus showing an increased cell perimeter and Feret's maximum diameter (1.6-fold, $p < 0.05$) (**Figure 3.1B,C**). Curiously, while Aβ treatment of 2 DIV reactive microglia promoted a prevalent ovoid shape with an enlarged cell area (1.9-fold, $p < 0.05$) (**Figure 3.1D**), 16 DIV cells exposed to Aβ show a more heterogeneous morphology with polarized microglia bearing one or two large processes, or a large lamellipodia with a thin process. In this case the cells show a reduction in cell perimeter and in Feret's maximum diameter (0.7-fold, $p < 0.05$), as well as an increased circularity (1.2-fold, $p < 0.05$) (**Figure 3.1E**). These morphometric alterations suggest that both young and aged cells increase soma volume, although the process shortening after Aβ treatment was not so evident in aged cells. However, whether these changes represent a similarly activated microglia or a separate functional state will be assessed in the next result sections.

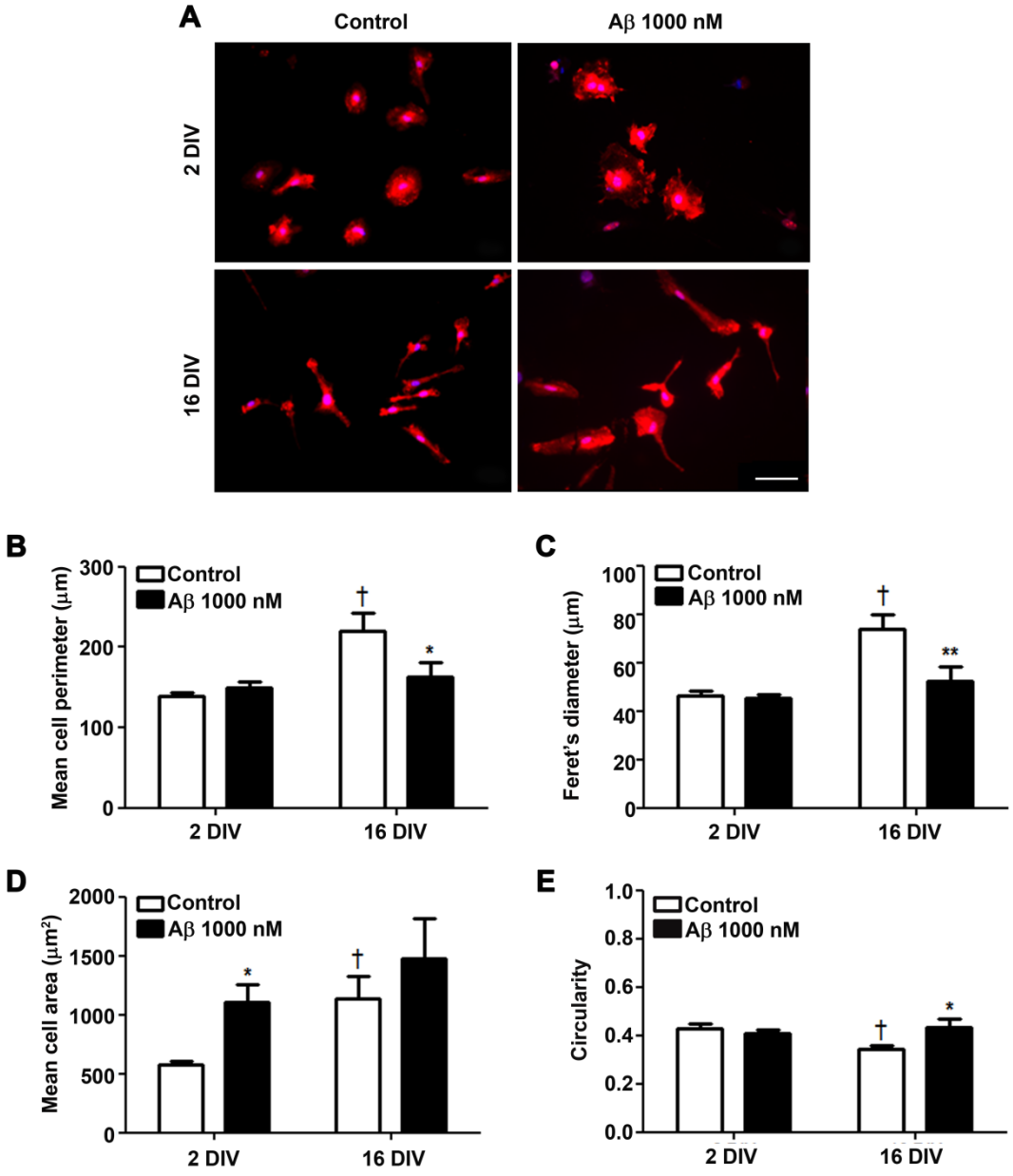

Figure 3. 1 – Reactive and aged cultured microglia evidence soma enlargement and amoeboid morphology after treatment with Aβ. Microglia that were kept in culture for 2 and 16 days *in vitro* (DIV) were treated with 1000 nM amyloid-β (Aβ) for 24 h. Cells were immunostained for Iba1 and characterized for their morphometric features. **(A)** Representative images show increased ramification by age, which is counteracted by Aβ exposure. Microglia perimeter **(B)**, Feret's diameter **(C)**, area **(D)** and circularity values **(E)** were measured using the computer program ImageJ and expressed in graph bars as mean ± SEM. Cultures, n = 4 per group. Two-way ANOVA (*Post-hoc* Bonferroni test): *$p < 0.05$ and **$p < 0.01$ *vs.* respective non-treated Control; †$p < 0.05$ *vs.* 2 DIV. Scale bar equals 50 µm.

3.3. Aβ differently upregulates the activation of MMP2 and MMP9 in reactive and aged cultured cells

Matrix metalloproteinases (MMPs) were shown to be important for the degradation of Aβ (Qiu et al., 1997) and MMP3, MMP12 and MMP13 to be activated by Aβ stimulation (Ito et al., 2007). In addition, MMP2 and MMP9 were found differently activated in animal models, AD patients and in *in vitro* microglia cultures, as well as in some studies related with the aggravation of AD disease (Brkic et al., 2015). Our data indicate that Aβ leads to increased activation of both MMP2 and MMP9 in the aged cells, while only stimulates MMP9 in the young reactive microglia, once no changes were detected for MMP2 activation (**Figure 3.2**). Data indicate that aged microglia by releasing these inflammatory components in the presence of Aβ may facilitate tau aggregation, but the dual roles of MMPs complicate the understanding of their beneficial or harmful effects in AD (Wang et al., 2014).

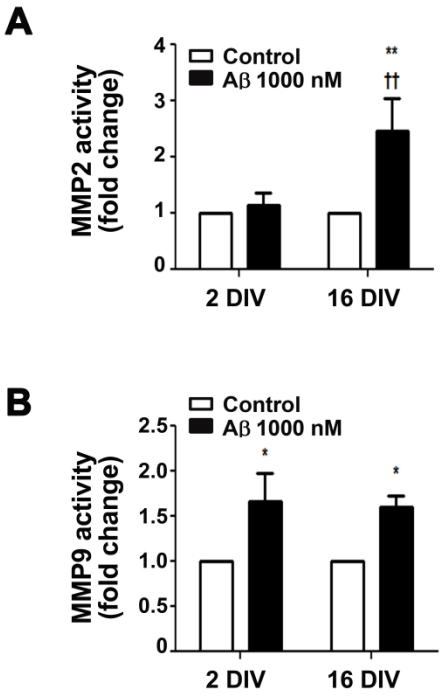

Figure 3. 2 – Release of metalloproteinase(MMP)-2 and MMP9 is differently induced by Aβ in reactive and aged cultured microglia. Microglia that were kept in culture for 2 and 16 days *in vitro* (DIV) were treated with 1000 nM amyloid-β (Aβ) for 24 h. Activities of MMP2 **(A)** and MMP9 **(B)** were evaluated by gelatin zymography. Results are expressed in graph bars as mean ± SEM. Cultures, $n = 4$ per group. Two-way ANOVA (*Post-hoc* Bonferroni test): $^{*}p < 0.05$ and $^{**}p < 0.01$ *vs.* respective non-treated Control; $^{††}p < 0.01$ *vs.* 2 DIV.

3.4. Aβ induces age-dependent changes in the autophagy-related Beclin-1 gene and LC3 puncta, and increase cellular senescence-associated biomarkers in 2 DIV microglia

Autophagy, or cellular self-digestion, is a highly regulated and evolutionarily conserved process that was shown to be impaired in AD (Zare-Shahabadi et al., 2015). We first evaluated microglia autophagic capacity by assessing the expression of Beclin-1, a protein known to be recruited to phagosomal membranes, and as participating in the early stages of autophagy and LC3-associated phagocytosis (Chifenti et al., 2013). We observed that Beclin-1 was upregulated by Aβ in young/activated 2 DIV cells (1.6-fold, $p < 0.05$, **Figure 3.3A**), but markedly decreased in the aged 16 DIV cells, with a slight and not significant increase upon Aβ stimulation. Based on the importance of LC3 processing for autophagosome formation, we next determined LC3-positive puncta indicative of autophagosome formation/accumulation (Klionsky et al., 2016). As indicated in **Figure 3.3B**, Aβ only slightly increased the number of 2 DIV cells presenting LC3-positive puncta. Intriguingly, the aged cells revealed to upregulate autophagy under Aβ treatment toward values of 2 DIV cells, either treated or untreated. Because Beclin-1 levels were shown to decline by aging (Shibata et al., 2006), we next assessed the cells that positively stained for senescence-associated beta-galactosidase (SA-β-gal) activity, a biomarker of cellular senescence (Sikora et al., 2011). We observed that the young/reactive microglia after being exposed to Aβ show increased cell staining for SA-β-gal (2.1-fold, $p < 0.05$, **Figure 3.3C**) very closely approaching the values of 16 DIV aged cells, where no further response was obtained for the presence of Aβ. To validate Aβ as an inducer of senescence-like response in 2 DIV microglial cells we additionally assessed changes in the expression of the microRNA (miR)-146a in both aged microglial cells. Actually, besides its numerous described functions and targets (Cardoso et al., 2016), miR-146a was reported to contribute to age-related dysfunction of macrophages (Jiang et al., 2012), and to loss of mitochondrial integrity and function in aged cells (Rippo et al., 2014). As anticipated, miR-146a increased expression was noticed in the 2 DIV microglia after treatment with Aβ, although values of 16 DIV cells were even superior (**Figure 3.3D**). These results suggest that Aβ promotes the formation of autophagosomes which turnover is impaired in aged microglia contributing to Aβ accumulation within these cells. In addition, Aβ switches the reactive 2 DIV microglia towards a senescent-like cell phenotype, probably compromising the response to stressors.

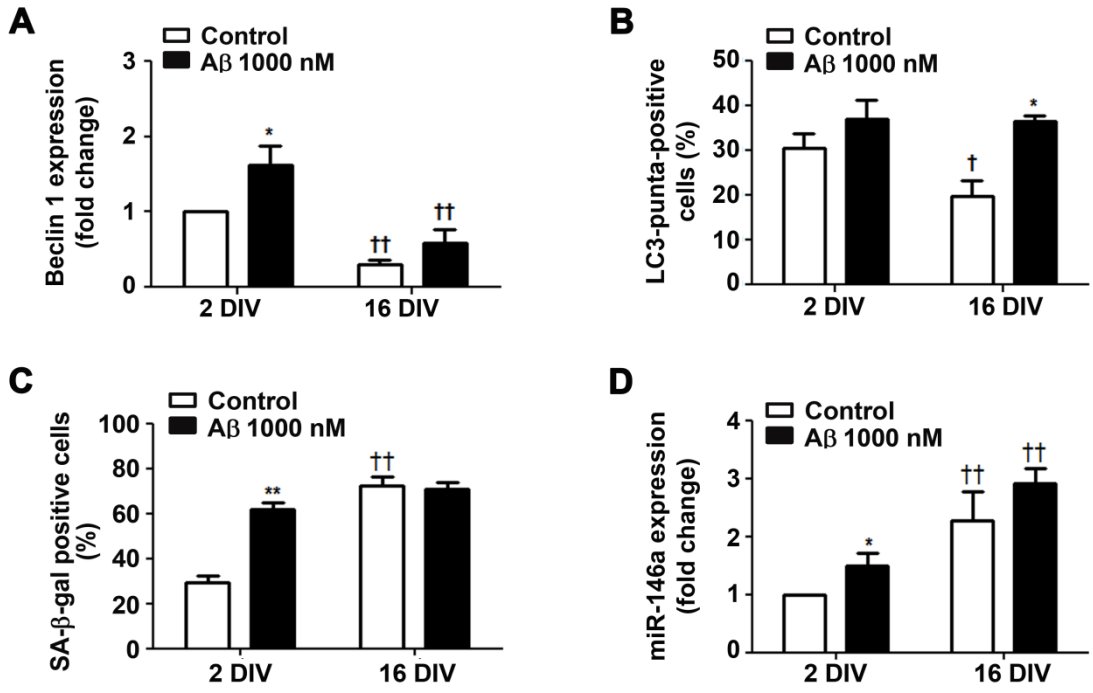

Figure 3. 3 - Autophagy and senescence are differently promoted by Aβ stressful stimulus in reactive and aged cultured microglia. Microglia that were kept in culture for 2 and 16 days *in vitro* (DIV) were treated with 1000 nM amyloid-β (Aβ) for 24 h. **(A)** Total cell lysates were analyzed for the presence of Beclin 1. **(B)** LC3-positive puncta cells were detected by immunostaining for LC3. **(C**) Activity of SA-β-gal was determined using a commercial kit, and SA-β-gal-positive cells were counted. **(D)** microRNA (miR)-146a expression was evaluated by RealTime PCR. Results are expressed in graph bars as mean ± SEM. Cultures, $n = 4$ per group. Two-way ANOVA (*Post-hoc* Bonferroni test): $^{*}p < 0.05$ and $^{**}p < 0.01$ *vs.* respective non-treated Control; $^{\dagger}p < 0.05$ and $^{\dagger\dagger}p < 0.01$ *vs.* 2 DIV.

3.5. Migration and phagocytic ability are impaired in the aged cultured microglia

Microglia migration dynamics together with synaptic pruning and phagocytic capacity of neuronal cells and their debris are part of their important functions in the central nervous system (Xavier et al., 2014; Zhang et al., 2016). Cell migration can be triggered by several chemoattractants, including ATP that when released by damaged neurons acts on $P2Y_{12}$ and $P2X_4$ receptors in microglia stimulating their migration (Miller and Stella, 2009). Our data show a higher fraction of migration for young cultured microglia as compared with the aged cells (**Figure 3.4**). In addition, while the 2 DIV cells positively respond to Aβ (1.7-fold, $p < 0.01$) and ATP chemotactic signals (2.0-fold, $p < 0.01$) the 16 DIV microglia failed to be attracted and to migrate towards either Aβ or ATP. These findings suggest that aging delays cell migration, a finding that is consistent with a compromised Aβ clearance by dysfunctional microglia.

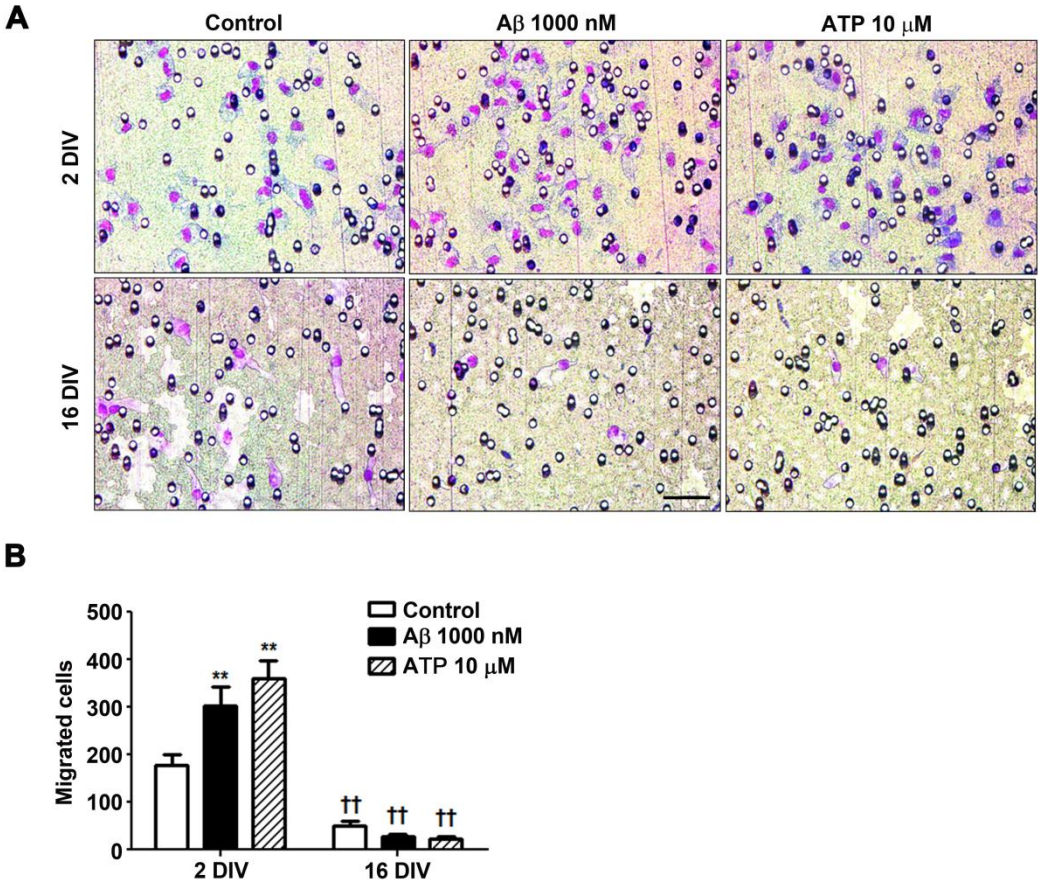

Figure 3. 4 – Activated 2 DIV microglia show increased migration ability towards Aβ and ATP, while the aged cultured microglia are immotile and irresponsive to such chemoattractants. Microglia were kept in culture for 2 and 16 days *in vitro* (DIV) and cellular chemotactic migration to 1000 nM amyloid-β (Aβ) and 10 μM ATP (positive chemotactic control) was evaluated after 6 h incubation using the Boyden chamber method. **(A)** Representative images of migrated cells to Aβ and ATP. **(B)** Number of migrated cells was counted and results are expressed in graph bars as mean ± SEM. Cultures, n = 4 per group. Two-way ANOVA (*Post-hoc* Bonferroni test): $^{**}p < 0.01$ *vs.* respective non-treated Control; $^{††}p < 0.01$ *vs.* 2 DIV. Scale bar equals 50 μm.

When microglia are activated they acquire an amoeboid phenotype and are able to exert phagocytosis, an important protective role for the efficient elimination of apoptotic cells and for neuronal circuitry reshape (Xavier et al., 2014). Our results corroborate previous data showing a reduction in phagocytosis when microglia are aged in culture (Caldeira et al., 2014). Newest results here presented show that 24 h incubation of reactive microglia with Aβ trigger a cellular response that equals that of aged cells, independently of being exposed or not to Aβ stimulus at this later time point, and represents a 2-fold reduction ($p < 0.01$) as depicted in **Figure 3.5A**. In addition, the number of cells able to engulf more than 10 beads was dramatically decreased upon Aβ interaction (10-fold reduction, $p < 0.01$, **Figure 3.5B**). Interestingly the number of 16 DIV

cells able to phagocytose 5-10 beads even further decrease in the presence of Aβ. These results suggest that Aβ reduces microglia phagocytosis in the reactive young cell toward the values of the aged and irresponsive microglia.

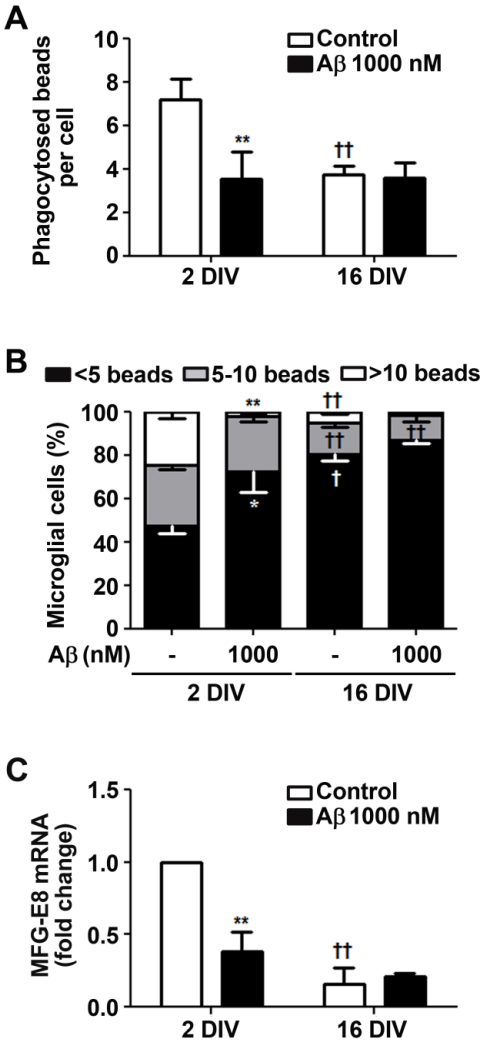

Figure 3. 5 – Aβ-treatment decreases the phagocytic ability of the activated 2 DIV microglia towards the low levels shown by the aged cultured cells. Microglia that were kept in culture for 2 and 16 days *in vitro* (DIV) were treated with 1000 nM amyloid-β (Aβ) for 24 h. Phagocytic capacity was assessed after 75 min incubation with fluorescent latex beads. **(A)** Number of phagocytozed beads per cell and **(B)** number of microglial cells phagocytosing less than 5, 5 to 10 and more than 10 beads was counted. **(C)** Expression of milk fat globule-EGF factor 8 protein (MFG-E8) that regulates microglial phagocytosis was evaluated by RealTime PCR. Results are expressed in graph bars as mean ± SEM. Cultures, $n = 4$ per group. Two-way ANOVA (*Post-hoc* Bonferroni test): $^{*}p < 0.05$ and $^{**}p < 0.01$ *vs.* respective non-treated Control; $^{\dagger}p < 0.05$ and $^{\dagger\dagger}p < 0.01$ *vs.* 2 DIV.

Milk Fat Globule Factor-E8 (MFG-E8) was shown to be released by microglia and to recognize phosphatidylserine “eat me” signals on the surface of apoptotic neurons and other cells, thus contributing to enhance microglia phagocytic properties (Fuller and

Van Eldik, 2008; Liu et al., 2013). Therefore, we decided to test whether Aβ was able to interfere with the expression of MFG-E8. Interestingly, we observed that treatment with Aβ triggers more than a 2-fold reduction ($p < 0.01$) in the expression of MFG-E8 in the 2 DIV microglia (**Figure 3.5C**). As before, the aged cells show downregulated expression of this phagocytic-related protein that was maintained after incubation with Aβ, reinforcing their irresponsive nature. We may conclude that despite the ability to migrate to sites of Aβ deposition the young activated microglia lose their phagocytosis capacity when facing Aβ, at least in primary cultures.

3.6. Aβ reduces the expression of inflammatory-related miR-155 and miR-124 in 2 DIV microglia

Recent studies indicate that miR-155 and miR-124a regulates T-cell functions during inflammation (Heyn et al., 2016). Both miRNAs are directly involved in microglia polarization, where miR-124 is considered to be associated with an anti-inflammatory phenotype, and miR-155 as having a determinant role in microglia activation (Ponomarev et al., 2013). To gain insight into the Aβ-induced alterations in microglia polarization we assessed the expression of these inflamma-miRNAs in the 2 DIV and 16 DIV microglia (**Figure 3.6**). Interestingly, we obtained a downregulated expression of both miR-155 and miR-124 by Aβ treatment in the 2 DIV microglial cells (0.7-fold and 0.6-fold, respectively, $p < 0.05$). Now considering the aged cultured microglia, no changes were obtained by the incubation of these cells with Aβ, probably due to the “dormancy” of these cells already showing an irresponsive phenotype with a reduced expression of either miR-155 or miR-124 (~0.5-fold as compared to the 2 DIV control microglia). These results suggest a direct repressor effect of Aβ in microglia polarization as M2 (miR-124) or M1 (miR-155) phenotypes in the 2 DIV microglia already activated by the isolation procedure. Thus, presence of mixed subpopulations and less responsive microglia subtypes following Aβ interaction should be envisaged as a consequence of this noxious stimulus.

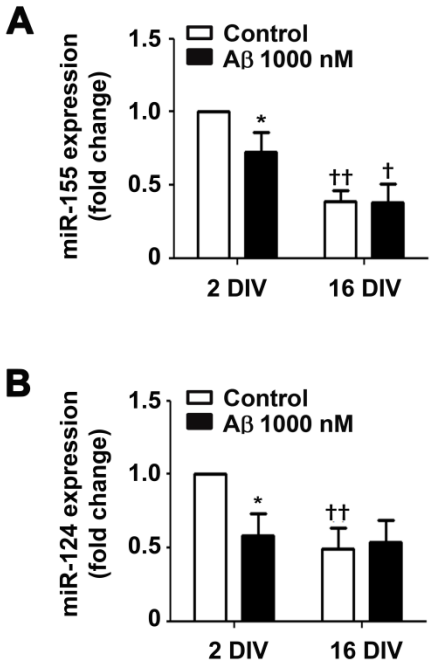

Figure 3. 6 – Aβ-treatment reduces the expression of miR-155 and miR-124 in the activated 2 DIV microglia towards the levels displayed by the aged cultured cells. Microglia that were kept in culture for 2 and 16 days *in vitro* (DIV) were treated with 1000 nM amyloid-β (Aβ) for 24 h. M1/pro-inflammatory-related miR-155 **(A)**, and M2/anti-inflammatory-related miR-124 **(B)** expression was evaluated by RealTime PCR. Results are expressed in graph bars as mean ± SEM. Cultures, $n = 4$ per group. Two-way ANOVA (*Post-hoc* Bonferroni test); $^{*}p < 0.05$ *vs.* respective non-treated Control; $^{\dagger}p < 0.05$ and $^{\dagger\dagger}p < 0.01$ *vs.* 2 DIV.

3.7. Aβ-treated 2 DIV microglia show phenotypic features of microglia activation, which are lacking in 16 DIV cells

To determine whether 2 DIV and 16 DIV microglia, despite the downregulation of miR-155 and miR-124 expression, were able to mount an inflammatory response upon Aβ insult we assessed a serial of commonly associated biomarkers of microglia activation. We started by evaluating the gene expression of the first line cytokines TNF-α, IL-1β and IL-6. We obtained a clear upregulation of all these pro-inflammatory cytokines in the young cultured 2 DIV cells (**Figure 3.7A**). The 16 DIV cells evidenced a 10-fold reduction in the mRNA expression of TNF-α, IL-1β and IL-6 as compared with the 2 DIV cells ($p < 0.01$). Curiously, even so, these cells showed to react to the exposure to 1000 nM Aβ by significantly increasing TNF-α and IL-1β gene expression, but not that of IL-6. Since we and others previously showed that high mobility group box-1 (HMGB1) is released by LPS-treated N9 microglia (Cunha et al., 2016) and promoted the synthesis of pro-IL-1β and pro-IL-18 (He et al., 2012), as well as the activation of NLRP3-inflammasome (Chi et al., 2015), we aimed to further explore these signaling pathways in our differently aged microglia model treated with Aβ. Interestingly, we observed a net

elevation of HMGB1 and IL-18 gene expression (1.6- and 2.1-fold, respectively, $p < 0.05$), but not of NLRP3 in the 2 DIV microglia exposed to Aβ. In contrast, significantly decreased levels were noticed in 16 DIV microglia ($p < 0.01$), which were almost unreactive to the stimulus (**Figure 3.7B**).

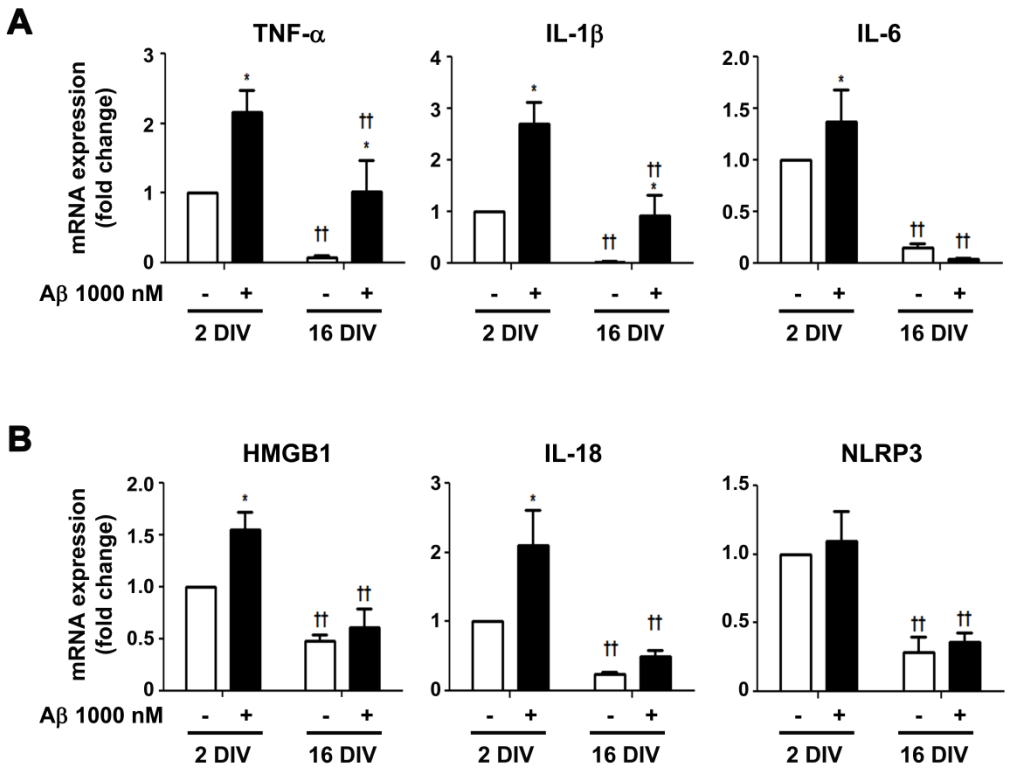

Figure 3. 7 – Aged cultured microglia only show some of the inflammatory mediators expressed by the 2 DIV cells upon Aβ-stressful stimulus. Microglia that were kept in culture for 2 and 16 days *in vitro* (DIV) were treated with 1000 nM amyloid-β (Aβ) for 24 h. The expression of inflammatory cytokines [e.g. tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6] **(A)** and inflammasome-related proteins [e.g. High-mobility group protein B1 (HMGB1), IL-18 and NOD-like receptor family pyrin domain containing 3 (NLRP3)] **(B)** was evaluated by RealTime PCR. Results are expressed in graph bars as mean ± SEM. Cultures, $n = 4$ per group. Two-way ANOVA (*Post-hoc* Bonferroni test): $^{*}p < 0.05$ *vs.* respective non-treated Control; $^{\dagger\dagger}p < 0.01$ *vs.* 2 DIV.

Surface Toll-like receptors are abundantly expressed in microglia and recruitment of TLR-2 and TLR-4 by HMGB1 and IL-1β was shown to amplify inflammation (Park et al., 2004; Facci et al., 2014). In accordance with previous results, Aβ triggered and enhanced expression of both TLR-2 and TLR-4 in 2 DIV, but not in 16 DIV cells (**Figure 3.8A,B**), which again revealed a suppressed basal expression of these receptors. The fractalkine/CX3CR1 signaling pathway was previously demonstrated to modulate microglial activation (Limatola and Ransohoff, 2014) and the receptor CX3CR1 to be associated with a less phagocytic and more inflammatory phenotype in in vivo AD

models (Merino et al., 2016). As documented for TLR-2 and TLR-4, a similar profile was obtained for the CX3XR1 expression in 2 DIV and 16 DIV aged microglia (**Figure 3.8C**), what reinforces the concept that only 2 DIV cells are able to sustain an inflammatory response upon Aβ exposure, and confirms the low responsiveness of 16 DIV microglia in conformity with a more irresponsive and senescent-like phenotype.

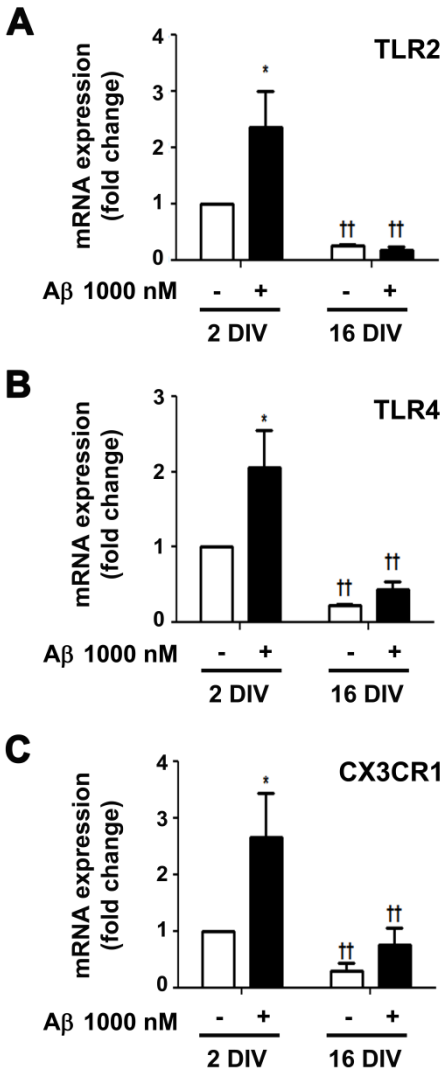

Figure 3. 8 – Aβ-treatment increases the expression of Toll-like receptor (TLR)2, TLR4 and CX3C chemokine receptor 1 (CX3CR1) i**n the activated 2 DIV microglia, but not in the aged cultured cells.** Microglia that were kept in culture for 2 and 16 days *in vitro* (DIV) were treated with 1000 nM amyloid-β (Aβ) for 24 h. Expression of TLR2 **(A)**, TLR4 **(B)** and CX3CR1 **(C)** was evaluated by RealTime PCR. Results are expressed in graph bars as mean ± SEM. Cultures, $n = 4$ per group. Two-way ANOVA (*Post-hoc* Bonferroni test): $^{*}p < 0.05$ *vs.* respective non-treated Control; $^{\dagger\dagger}p < 0.01$ *vs.* 2 DIV.

3.8. Imbalance of M1 and M2 phenotypes in 2 DIV and 16 DIV microglia suggests dysregulation of microglia activation by Aβ incubation

From previous data, the elevation on IL-1β expression, mainly in 2 DIV microglia, indicates that the cell assumes preferentially the M1 phenotype upon Aβ exposure. However, the CX3CR1 increased expression also indicates microglia subsets with M2a polarization (Chhor et al., 2013). Therefore, we decided to further characterize the microglia phenotypes in 2 DIV and 16 DIV exposed to Aβ by evaluating additional M1 and M2 markers. For that we first evaluated mRNA expression of inducible nitric oxide synthase (iNOS) and major histocompatibility (MHC) class II, which are considered M1/pro-inflammatory microglia markers, although MHCII has also been attributed to M2b polarized macrophages (Roszer, 2015). As shown in **Figure 3.9A**, while iNOS is highly induced by Aβ both in young and aged microglia (4.7- and 5.4-fold for 2 and 16 DIV, $p < 0.05$ and $p < 0.01$, respectively), MHC class II is only markedly increased in young cultured cells (11.4-fold, $p < 0.01$), suggesting a preferential M1 polarization in 2 DIV cells and a mixture of phenotypes in the 16 DIV microglia. Then, we proceded for the characterization of mRNA expression of M2/anti-inflammatory microglia markers, such as Arginase 1 (prevalent in M2a activation state) and transforming growth factor-β (TGF-β) [suggested to be increased in the M2a/M2c/M2d subtypes (Roszer, 2015)]. As observed in **Figure 3.9B**, Arginase 1, considered to be a repair/regenerative gene (Chhor et al., 2013), is only increased by Aβ in young cultured cells (2.6-fold, $p < 0.05$), with levels that, although slightly elevated upon Aβ, represented in the 16 DIV cells less than 40% ($p < 0.01$) of those in 2 DIV cells. In what concerns TGF-β expression, with neuroprotective and pro-survival properties (Dobolyi et al., 2012; Ryu et al., 2012), both young and aged Aβ-treated cells evidenced an increased expression (2.4- and 2.3-fold, respectively, $p < 0.05$). Overall these results suggest that both M1 and M2 subpopulations are present upon Aβ treatment. However while 2 DIV cells mainly express M1 markers, a phenotypic dysregulation with overlapping of microglial M1 and M2 markers is more the signature of the aged microglia. These cells additionally showed a decreased ability in mounting an adequate inflammatory response when stressed with Aβ.

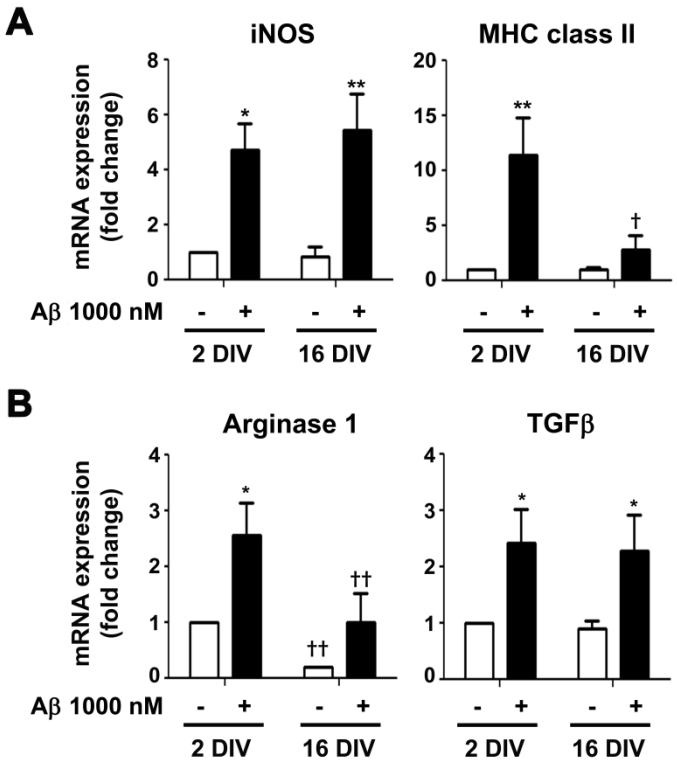

Figure 3. 9 – Aβ-treatment triggers an age-dependent shift in M1/pro-inflammatory and M2/anti-inflammatory polarization markers. Microglia that were kept in culture for 2 and 16 days *in vitro* (DIV) were treated with 1000 nM amyloid-β (Aβ) for 24 h. The expression of M1/pro-inflammatory [e.g. inducible nitric oxide synthase (iNOS) and major histocompatibility (MHC) class II] **(A)** and M2/anti-inflammatory [e.g. Arginase and transforming growth factor-β (TGF-β)] **(B)** were evaluated by RealTime PCR. Results are expressed in graph bars as mean ± SEM. Cultures, *n* = 4 per group. Two-way ANOVA (*Post-hoc* Bonferroni test): **p* < 0.05 and ***p* < 0.01 *vs.* respective non-treated Control; †*p* < 0.05 and ††*p* < 0.01 *vs.* 2 DIV.

3.9. Proportion of CD11b and CD86 positive microglia differs between 2 DIV and 16 DIV cells after incubation with Aβ

To further understand whether the lower reactivity of 16 DIV microglia towards Aβ was associated with an increased expression of CD86 which as previously indicated to be age-related (Kohman et al., 2013), we evaluated changes in the proportion of the M1 markers CD11b+ (co-stimulatory ligand) and CD86+ (integrin αM) cells, after Aβ stimulus, in our microglia aged model of 2 DIV and 16 DIV by flow cytometry. As depicted in **Figure 3.10A**, the naïve aged microglia showed a decreased number of CD11b+ cells when compared to young/activated 2 DIV cells. In addition, these aged cells revealed to have an increased number of CD11b-/CD86- (~50%), together with elevated proportions of mixed CD11b-/CD86+ and CD11b+/CD86+ populations, than those of 2 DIV microglia, corroborating the aging-like profile status of 16 DIV microglia (**Figures 3.10B,C, Table 3.4**). When treated with Aβ both 2 DIV and 16 DIV cells showed a decreased population of CD11b+ cells. While 2 DIV microglia shifted from medium to high density of CD11b-

/CD86-, the number of CD11b-/CD86+ in 16 DIV cells increased 4-fold (~25%) upon Aβ treatment and represent a 24-fold increase as compared with their 2 DIV counterparts ($p < 0.01$). No relevant changes were noticed when we assessed the expression of both CD11b and CD45 (data not shown). These results further highlight that *in vitro* aging reduces CD11b+ microglia reactivity, while indicate a gain of function of 16 DIV cells to co-stimulate other immune cells through CD86 signaling, namely in the presence of Aβ.

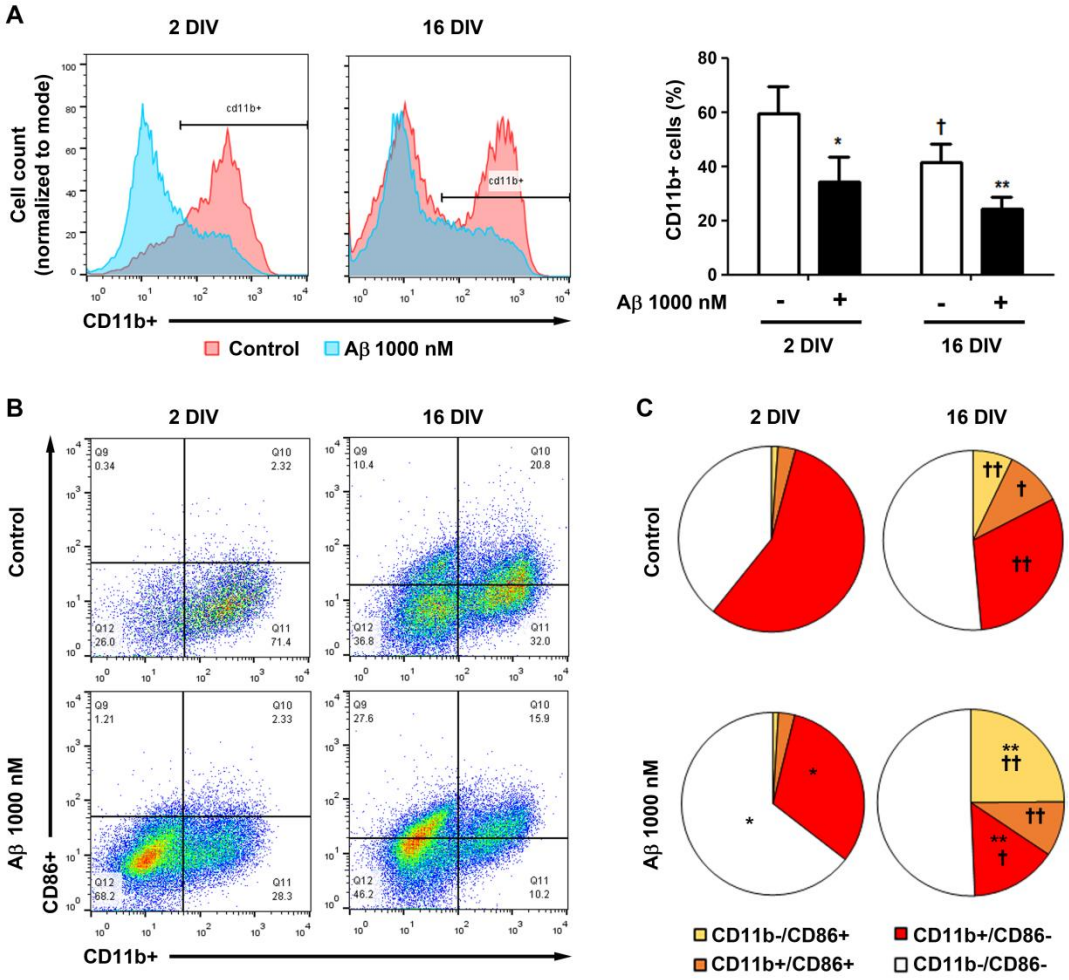

Figure 3. 10 – Microglia CD11b+ cells decrease with aging and Aβ-treatment contrasting with CD86+ cells whose prevalence increase by the same conditions. Microglia that were kept in culture for 2 and 16 days *in vitro* (DIV) were treated with 1000 nM amyloid-β (Aβ) for 24 h. The population of CD11+ and CD86+ cells was detected by flow cytometry. **(A)** Analysis of microglia expressing CD11+. Results are expressed in graph bars as mean ± SEM. Cultures, $n = 4$ per group. Two-way ANOVA (*Post-hoc* Bonferroni test): $^{*}p < 0.05$ and $^{**}p < 0.01$ *vs.* respective non-treated Control; $^{\dagger}p < 0.05$ *vs.* 2 DIV. **(B)** Representative flow cytogram of CD11+ and CD86+ cells in the population of 2 DIV activated and aged cultured microglia. **(C)** Results are expressed in 2D pie graphs as mean. Cultures, $n = 4$ per group. Two-way ANOVA (*Post-hoc* Bonferroni test): $^{*}p < 0.05$ and $^{**}p < 0.01$ *vs.* respective non-treated Control; $^{\dagger}p < 0.05$ and $^{\dagger\dagger}p < 0.01$ *vs.* 2 DIV.

Table 3. 4 – Flow cytometry analysis of the expression of CD11b and CD86 in microglia

	2 DIV		16 DIV	
	Control	**Aβ**	**Control**	**Aβ**
CD11b-/CD86+	1.15 (±0.72)	0.96 (±0.15)	7.16 (±3.06)	24.87 (±6.45) *††
CD11b+/CD86+	3.08 (±0.71)	2.88 (±0.21)	10.26 (±4.17) †	9.51 (±2.32) ††
CD11b+/CD86-	56.56 (±6.68)	31.64 (±5.87) *	31.12 (±3.48) ††	14.95 (±2.64) **
CD11b-/CD86-	39.21 (±9.54)	64.52 (±9.03) *	51.46 (±8.90)	50.67 (±9.59)

All results are means ± SEM from at least four independent experiments. Microglial cells were kept in culture for 2 days *in vitro* (DIV) and 16 DIV and treated with amyloid-β (Aβ) at 1000 nM for 24 h. The population of CD11+ and CD86+ expressing microglia was detected by flow cytometry using specific antibodies. Two-way ANOVA (*Post-hoc* Bonferroni test) *$p < 0.05$, **$p < 0.01$ *vs.* respective Control; †$p < 0.05$, ††$p < 0.01$ *vs.* 2 DIV.

4. Discussion

In the present manuscript we used an *in vitro* aging model developed in our laboratory (Caldeira et al., 2014) to assess whether activated microglia at 2 DIV (as a consequence of the isolation procedure) and mature microglia at 16 DIV (aged in culture) will differently react to Aβ stimulation. Our purpose was to better realize the complexity of microglia activation and cell malfunction processes in AD and the association of immunosenescence to AD emergence, as an attempt to understand the existence of different microglia polarized phenotypes along the progression of the disease and the controversial results published so far about the role of subacute neuroinflammation in AD. Actually, AD pathophysiology is overlaid by the aging effects on the CNS, and microglia were shown to be dysfunctional in aging and AD (Mosher and Wyss-Coray, 2014; Cykowski et al., 2016).

Here, we observed that several neuroprotective functions, namely phagocytosis and migration abilities, as well as autophagy, are impaired by *in vitro* aging. Furthermore, Aβ-induced inflammatory response is also reduced in aged 16 DIV cells with compromised expression of inflamma-miRs and typical CD11b marker, but enhanced expression of the co-stimulatory CD86 molecule. In addition, our data show that Aβ is a stressor-inducer of microglia senescence based on the characteristics displayed by the activated cells upon interaction with Aβ. Our data is not without precedent since the population of senescent astrocytes was previously shown to increase in human brain during aging and AD (Bhat et al., 2012), and dystrophic microglia was found in the brain of AD patients (Streit et al., 2009). Results are supportive that not only aged microglia have compromised function, but also that Aβ treatment reduces the ability of the activated

microglia to fully develop neuroprotective and inflammatory reaction towards the noxious Aβ stimulus.

In accordance with our previous data showing that the impaired cell function by *in vitro* aging is not associated with loss of cell viability (Caldeira et al., 2014), we did not observe age-dependent changes in cell death, either in the absence or in the presence of Aβ treatment, reinforcing the validity of our *in vitro* model to evaluate cell signaling perturbing effects by aging and Aβ. Changes in microglia morphology are associated with different functional states, where activation relates with larger somata and shorter processes that progress to an amoeboid morphology with downregulation of the ramified phenotype, characteristic of the surveilling microglia (Harry, 2013). Moreover, aged microglia also revealed to be smaller and less branched and less effective in mounting a normal response to injury. These cells with dystrophic appearance and less capacity to phagocytose and migrate, probably due to intracellular oxidative stress, were indicated to be senescent (Streit et al., 2008). We previously demonstrated that cells acutely isolated and maintained for 2 DIV in culture behave as activated microglia, while when maintained in culture for 16 DIV the cells exhibit a more bipolar shape and shorter large processes (Caldeira et al., 2014). Here, we further identified aged cells with altered nuclei morphology showing a thin and elongated shape. This type of cells commonly called as rod cells, have been associated to chronically inflamed cerebral cortex (Hof and Mobbs, 2009) and acutely dementing processes (Graeber, 2010). Curiously, when these cells were exposed to Aβ they shift to an amoeboid morphology, more notoriously in the 2 DIV stimulated microglia, a morphometric alteration characteristic of a reactive microglia (Nakajima and Kohsaka, 2004), thus suggesting that Aβ causes cell shape alterations compatible with an activated cell. To note, however, that the aged 16 DIV cells evidence the presence of microglia morphological subclasses, a finding also recently observed in the hippocampus of AD patients (Bachstetter et al., 2015).

Activation of MMPs has been implicated in AD pathogenesis, playing an important role in Aβ degradation (Miners et al., 2011). Here we show that the 2 DIV cells only secrete MMP9, and not MMP2, upon Aβ treatment. Surprisingly, the aged cells by secreting increased levels of MMP2 and MMP9 reveal to be somehow activated by the Aβ stimulus. MMP2 was suggested to be the major protective gelatinase in AD and to be overexpressed in astrocytes surrounding senile plaques in the transgenic mice brain, while MMP9 expression is considered a characteristic of AD with a potential neurotoxic side effect (Wang et al., 2014). Indeed, expression and higher activation of MMP9, but not MMP2, were reported in serum and brain samples of mild cognitive impairment (MCI)

and AD patients (Lorenzl et al., 2008; Bruno et al., 2009). Our data reinforce the role of microglia in MMPs release following Aβ insult highlighting an increased expression with age.

Microglia migration is an important feature of the activated cell (Kettenmann et al., 2011). As in our previous study (Caldeira et al., 2014), we show that aged microglia is significantly less susceptible to ATP-induced chemotactic signals. In *ex vivo* retinal explants, the motility process in microglia obtained from aged mice (18 to 24 months of age) was also reduced relatively to the young adult animal cells (2 to 3 months of age) (Damani et al., 2011). A recent study similarly reported that the intranasally, or intravenously, administered microglia to mice is only lately detected if derived from young donors (Leovsky et al., 2015), further reinforcing microglia migration impairment with age. Young microglia, but not the aged ones, revealed to also migrate towards Aβ, an effect probably related with the release of ATP promoted by both fibrillar and oligomeric $A\beta_{1-42}$ species (Kim et al., 2012). Functional impairment of microglia was likewise shown to coincide with Aβ deposition in a mice model of AD due to a decline in their phagocytic capacity (Krabbe et al., 2013). Actually, microglial-mediated clearance of tissue debris was demonstrated to decay with aging (Neumann et al., 2009), to be compromised in older AD animal models (Njie et al., 2012), and to be associated with immunosenescence (Li, 2013). In our microglia *in vitro* aging the phagocytic ability of 2 DIV microglia was reduced by Aβ, in terms of beads per cell and maximum amount in each cell, to levels close of the 16 DIV aged cells. Microglia phagocytosis is also related with the recognition of phosphatidylserine receptors following docking of the MFG-E8 molecule (Li, 2012), which was also downregulated by aging and Aβ in our model. Reduction of phagocytic efficiency could arise from decreased chemotactic responses, but also from impairment in the detection of targets caused by downregulation of certain receptors (Mosher and Wyss-Coray, 2014). This is not the case of TLR-2 and TLR-4 that revealed to be enhanced in young cells and upon Aβ interaction. However, they showed marked reduced levels in the aged cells, probably accounting for impaired phagocytosis and migration of the older cells.

Numerous studies support the pivotal role of miRNAs in the regulation of microglial phenotype by promoting microglial quiescence (miR-124), or by driving microglial inflammatory and immune responses (miR-155 and miR-146a) (Ponomarev et al., 2013). While miR-124 was shown to be downregulated in hippocampal brain samples of AD patients ranging from early to severe disease stages (Lukiw, 2007), miR-155 was reported to be overexpressed in circulating fluids and cells of AD individuals (Alexandrov

et al., 2012; Guedes et al., 2016), as well as in the brain of the 3xTg-AD mice model (Guedes et al., 2014). However, in other works miR-155 expression was found significantly reduced in old individuals (Noren Hooten et al., 2010). Our results indicate that both miR-124 and miR-155 are decreased in 16 DIV cells, but that their downregulation in 2 DIV microglia is also a result of Aβ interaction. Such reduction may have important consequences in AD progression since miR-155 was recently proposed to have a protective role as an anti-inflammatory factor (Li et al., 2016).

Despite inflammatory-miRNA reduction in the presence of Aβ, the gene expression of pro-inflammatory cytokines, HMGB1 and IL-18, but not NLRP3, increased in 2 DIV cells upon Aβ treatment, while only TNF-α and IL-1β were enhanced in the 16 DIV cells. The understanding of the pro-inflammatory cytokines signaling pathways associated to AD is crucial to dissect their beneficial and harmful effects on AD progression and to define the relevance of using NSAIDs. Our data suggest, that neuroinflammation by aged microglia is at a much lower level than that of the activated and recently isolated cells leading to questioning the usefulness of NSAIDs in AD late stages. However, further studies are required to decide on the existence of different inflammation-associated stages during AD progression and the different therapeutic approaches that should be accomplished. Indeed, based on our data, M1 polarized 2 DIV microglia are much less prevalent in the aged microglia. The increase of TNF-α and IL-1β in AD, a consensual concept and suggested to be implicated in AD pathogenesis (Wang et al., 2015), are here manifested as targets for selective tuning, once are transversal to both 2 DIV and 16 DIV microglia. Relatively to the other inflammatory-related mediators in the pathogenesis of AD they remain still elusive, mainly when considering the aging effects, and need additional investigations. Relatively to IL-6, also indicated to be elevated in AD, it is important to note that this cytokine is released by cells other than microglia, such neurons and astrocytes, and dual effects with an important role in controlling inflammation and promoting neurogenesis. Aged microglia clearly showed to produce low levels, which even slightly decrease with Aβ. Relationship between IL-6 concentrations and aging are not clearly established, and although suggested to be increased, no identification of the donor cell is provided and conflicting results have been produced (Maggio et al., 2006). We evidenced that Aβ and LPS trigger the release of HMGB1 from microglia. HMGB1, a nuclear protein acting as a co-factor for gene transcription, is considered a pro-inflammatory cytokine that signals trough TLR2 and TLR4 when released into the extracellular fluid (Park et al., 2004). HMGB1 was lately showed to induce neurite degeneration and to be involved in AD pathology (Fujita et al., 2016). The molecule is more frequently indicated to act on microglia to mediate

neuroinflammation (Gao et al., 2011) than to be released by the stimulated cell (Sun et al., 2014). Anyway, young activated microglia is more prone in secreting HMGB1 than the aged cells after Aβ exposure, what also relates with the low serum levels observed in aged individuals (Fu et al., 2016). TLR2 and TLR4 showed the same expression profile suggesting that their activation may result from the HMGB1 probably released to the cell milieu. Although IL-18 has been indicated to be produced downstream of NLRP3 (Zaki et al., 2010), it was recently associated to NLRP1 inflammasome, as well (Murphy et al., 2016). Our results do not sustain NLRP3 activation, although clearly show upregulated IL-18, again more notoriously in the 2 DIV cells than in 16 DIV cells. In a recent paper both inflammasome components were indicated to be activated in AD, but their direct association with microglia was not accomplished (Saresella et al., 2016). Most interesting, dendritic cells from elderly mice not only revealed defective NLRP3 activation, but also increased expression of pro-IL-18, when compared to young infected mice with influenza virus, highlighting impaired inflammasome signaling pathway by aging. Relatively to the increased expression of CX3CR1 in the 2 DIV cells activated with Aβ it sustains the less phagocytic and the inflammatory phenotype of these cells, as observed in in vivo AD models (Merino et al., 2016). Similar increase was not noticed in the 16 DIV cells, corroborating their less responsive phenotype as previously observed for the above mentioned inflammatory biomarkers and also found in aged mice (Wynne et al., 2010).

CX3CR1 increased expression and secretion of TGFβ are related with the M2 polarization, which is thought to down-regulate M1-mediated inflammatory responses (Chhor et al., 2013; von Bernhardi et al., 2015). However, there are indications that M2 cytokines are also associated with a chronic inflammatory process status. Our data further suggest that we may have a mixture of M1 and M2 phenotypes in both different aged cells populations, although with a high prevalence of the M1 phenotype in the 2 DIV cells. Actually M1 and M2 phenotypes are the extreme subtypes and the existence of different heterogeneous activation states, which have been later documented in aging and AD (Bachstetter et al., 2015; Grabert et al., 2016) reflecting the plastic nature of microglia. Increase of iNOS and MHC class II are more associated with the M1 phenotype while that of Arginase 1 and TGFβ are associated with the M2 subtype (Chhor et al., 2013). Nevertheless, MHC class II has also been attributed to M2 polarized macrophages (Roszer, 2015). Increased microglial iNOS and TGFβ signaling by aging and Aβ was previously documented (Dheen et al., 2005; Doyle et al., 2010; Mosher and Wyss-Coray, 2014; von Bernhardi et al., 2015). These findings may help to justify why

we found elevation of TGFβ and iNOS gene expression elevation in the aged cells treated with Aβ, when all the other tested biomarkers were found almost unchanged.

Further accounting for the presence of heterogeneous microglia populations mainly in the 16 DIV microglia, are the existence of four separate types of microglia, i.e CD11b-/CD86-, CD11b-/CD86+, Cd11b+/CD86- and Cd11b+/CD86+, differently represented in 2 DIV microglia and 16 DIV microglia. These distinct subsets may derive from dissimilarities in the differentiation stages that may contribute to morphological and functional diversities, as well as for discrepancies that are often found in the literature. Major differences induced by Aβ are the decrease in CD11b+ cells in 2 DIV and the increase in CD86+ cells in 16 DIV microglia. This age effect is in line with previous studies showing that aged mice have a higher proportion of CD86+ microglia (Kohman et al., 2013). CD86, usually expressed by antigen-presenting cells, acts as a co-stimulatory molecule for T cell activation (Tambuyzer et al., 2009). Actually, microglia showed to be efficient Aβ antigen-presenting cells upon interferon-γ stimulation, supporting CD86-dependent Aβ-reactive T cell activation (Monsonego et al., 2003). Our results corroborate that aging and Aβ may potentiate interactions between microglia and infiltrating T cells, thus concurring for immune dysfunction. Interestingly, the M2b polarized microglia with either pro- or anti-inflammatory functions and associated with the recruitment of regulatory T cells show high MHC class II and CD86 expression, what seems to preferentially occur in our aged Aβ-treated cells. In addition, loss of 2 DIV CD11b, also known as αMβ2 integrin, by Aβ interaction may correlate with the decreased microglia phagocytic and migration abilities. Indeed, integrin αMβ2 is reported to be implicated in phagocytosis, cell-mediated killing, chemotaxis and cellular activation (Cougoule, et al. 2004; Chen, et al. 2008).

5. Conclusions

Data show that microglia activation by Aβ depends on the polarization state of the cell. If already activated, microglia react with increased migration and expression of all major inflammatory biomarkers (except NLRP3), but also showing dysfunctional consequences as low phagocytic ability, increased senescence-like behavior, decreased CD11b immunoreactivity and reduced inflammatory miR-155 and miR-124. Changes are much less notorious in the already mature/aged microglia that only respond by activation of MMP2 and MMP9, increased LC3-punta and CD86 immunostaining, together with elevated iNOS, TGFβ and TNF-α gene expression. In addition, the distribution of M1 and

M2 polarized markers indicate that the 2 DIV cells assume a predominant M1 phenotype contrasting with that of 16 DIV cells showing diverse microglia subtypes that include M2 subclasses. Overall, our results point to age-related behavioral alterations suggestive to differently influence AD initiation and progression.

6. References

Alexandrov, P.N., Dua, P., Hill, J.M., Bhattacharjee, S., Zhao, Y., and Lukiw, W.J. (2012). microRNA (miRNA) speciation in Alzheimer's disease (AD) cerebrospinal fluid (CSF) and extracellular fluid (ECF). Int J Biochem Mol Biol 3, 365-373.

Bachstetter, A.D., Van Eldik, L.J., Schmitt, F.A., Neltner, J.H., Ighodaro, E.T., Webster, S.J., Patel, E., Abner, E.L., Kryscio, R.J., and Nelson, P.T. (2015). Disease-related microglia heterogeneity in the hippocampus of Alzheimer's disease, dementia with Lewy bodies, and hippocampal sclerosis of aging. Acta Neuropathol Commun 3, 32. doi: 10.1186/s40478-015-0209-z

Barateiro, A., Miron, V.E., Santos, S.D., Relvas, J.B., Fernandes, A., Ffrench-Constant, C., and Brites, D. (2013). Unconjugated bilirubin restricts oligodendrocyte differentiation and axonal myelination. Mol Neurobiol 47, 632-644. doi: 10.1007/s12035-012-8364-8

Barateiro, A., Vaz, A.R., Silva, S.L., Fernandes, A., and Brites, D. (2012). ER Stress, Mitochondrial Dysfunction and Calpain/JNK Activation are Involved in Oligodendrocyte Precursor Cell Death by Unconjugated Bilirubin. Neuromolecular Med. doi: 10.1007/s12017-012-8187-9

Bhat, R., Crowe, E.P., Bitto, A., Moh, M., Katsetos, C.D., Garcia, F.U., Johnson, F.B., Trojanowski, J.Q., Sell, C., and Torres, C. (2012). Astrocyte senescence as a component of Alzheimer's disease. PLoS One 7, e45069. doi: 10.1371/journal.pone.0045069

Birch, A.M., Katsouri, L., and Sastre, M. (2014). Modulation of inflammation in transgenic models of Alzheimer's disease. J Neuroinflammation 11, 25. doi: 10.1186/1742-2094-11-25

Brkic, M., Balusu, S., Libert, C., and Vandenbroucke, R.E. (2015). Friends or Foes: Matrix Metalloproteinases and Their Multifaceted Roles in Neurodegenerative Diseases. Mediators of Inflammation. doi: 10.1155/2015/620581

Bruno, M.A., Mufson, E.J., Wuu, J., and Cuello, A.C. (2009). Increased matrix metalloproteinase 9 activity in mild cognitive impairment. J Neuropathol Exp Neurol 68, 1309-1318. doi: 10.1097/NEN.0b013e3181c22569.

Caldeira, C., Oliveira, A.F., Cunha, C., Vaz, A.R., Falcão, A.S., Fernandes, A., and Brites, D. (2014). Microglia change from a reactive to an age-like phenotype with the time in culture. Front Cell Neurosci 8, 152. doi: 10.3389/fncel.2014.00152

Cardoso, A.L., Guedes, J.R., and De Lima, M.C. (2016). Role of microRNAs in the regulation of innate immune cells under neuroinflammatory conditions. Curr Opin Pharmacol 26, 1-9. doi: 10.1016/j.coph.2015.09.001

Chen, J., Namiki, S., Toma-Hirano, M., Miyatake, S., Ishida, K., Shibata, Y., et al. (2008). The role of CD11b in phagocytosis and dendritic cell development. Immunol Lett 120:42-48. doi:10.1016/j.imlet.2008.06.010

Chhor, V., Le Charpentier, T., Lebon, S., Ore, M.V., Celador, I.L., Josserand, J., Degos, V., Jacotot, E., Hagberg, H., Savman, K., Mallard, C., Gressens, P., and Fleiss, B. (2013). Characterization of phenotype markers and neuronotoxic potential of polarised primary microglia in vitro. Brain Behav Immun 32, 70-85. doi: 10.1016/j.bbi.2013.02.005

Chi, W., Chen, H., Li, F., Zhu, Y., Yin, W., and Zhuo, Y. (2015). HMGB1 promotes the activation of NLRP3 and caspase-8 inflammasomes via NF-kappaB pathway in acute glaucoma. J Neuroinflammation 12, 137. doi: 10.1186/s12974-015-0360-2

Chifenti, B., Locci, M.T., Lazzeri, G., Guagnozzi, M., Dinucci, D., Chiellini, F., Filice, M.E., Salerno, M.G., and Battini, L. (2013). Autophagy-related protein LC3 and Beclin-1 in the first trimester of pregnancy. Clin Exp Reprod Med 40, 33-37. doi: 10.5653/cerm.2013.40.1.33

Colton, C.A., Mott, R.T., Sharpe, H., Xu, Q., Van Nostrand, W.E., and Vitek, M.P. (2006). Expression profiles for macrophage alternative activation genes in AD and in mouse models of AD. J Neuroinflammation 3, 27. doi: 10.1186/1742-2094-3-27

Cougoule, C., Wiedemann, A., Lim, J., and Caron, E. (2004). Phagocytosis, an alternative model system for the study of cell adhesion. Semin Cell Dev Biol 15:679-689. doi:10.1016/j.semcdb.2004.09.001

Cristóvão, A.C., Saavedra, A., Fonseca, C.P., Campos, F., Duarte, E.P., and Baltazar, G. (2010). Microglia of rat ventral midbrain recovers its resting state over time in vitro: let microglia rest before work. J Neurosci Res 88, 552-562. doi: 10.1002/jnr.22219

Cunha, C., Gomes, C., Vaz, A.R., and Brites, D. (2016). Exploring New Inflammatory Biomarkers and Pathways during LPS-Induced M1 Polarization. Mediators Inflamm 2016, 6986175. doi: 10.1155/2016/6986175.

Cykowski, M.D., Takei, H., Van Eldik, L.J., Schmitt, F.A., Jicha, G.A., Powell, S.Z., and Nelson, P.T. (2016). Hippocampal Sclerosis but Not Normal Aging or Alzheimer Disease Is Associated With TDP-43 Pathology in the Basal Forebrain of Aged Persons. J Neuropathol Exp Neurol 75, 397-407. doi: 10.1093/jnen/nlw014

Damani, M.R., Zhao, L., Fontainhas, A.M., Amaral, J., Fariss, R.N., and Wong, W.T. (2011). Age-related alterations in the dynamic behavior of microglia. Aging Cell 10, 263-276. doi: 10.1111/j.1474-9726.2010.00660.x

Daniels, M.J., Rivers-Auty, J., Schilling, T., Spencer, N.G., Watremez, W., Fasolino, V., Booth, S.J., White, C.S., Baldwin, A.G., Freeman, S., et al. (2016). Fenamate NSAIDs inhibit the NLRP3 inflammasome and protect against Alzheimer's disease in rodent models. Nat Commun 7, 12504. doi: 10.1038/ncomms12504

Dheen, S.T., Jun, Y., Yan, Z., Tay, S.S., and Ling, E.A. (2005). Retinoic acid inhibits expression of TNF-alpha and iNOS in activated rat microglia. Glia 50, 21-31. doi: 10.1002/glia.20153

Dobolyi, A., Vincze, C., Pal, G., and Lovas, G. (2012). The neuroprotective functions of transforming growth factor beta proteins. Int J Mol Sci 13, 8219-8258. doi: 10.3390/ijms13078219

Doyle, K.P., Cekanaviciute, E., Mamer, L.E., and Buckwalter, M.S. (2010). TGFbeta signaling in the brain increases with aging and signals to astrocytes and innate immune cells in the weeks after stroke. J Neuroinflammation 7, 62. doi: 10.1186/1742-2094-7-62

Facci, L., Barbierato, M., Marinelli, C., Argentini, C., Skaper, S.D., and Giusti, P. (2014). Toll-like receptors 2, -3 and -4 prime microglia but not astrocytes across central nervous system regions for ATP-dependent interleukin-1beta release. Sci Rep 4, 6824. doi: 10.1038/srep06824

Falcão, A.S., Carvalho, L.A., Lidonio, G., Vaz, A.R., Lucas, S.D., Moreira, R., and Brites, D. (2016). Dipeptidyl vinyl sulfone as a novel chemical tool to inhibit HMGB1/NLRP3-inflammasome and inflamma-miRs in Abeta-mediated microglial inflammation. ACS Chem Neurosci. doi: 10.1021/acschemneuro.6b00250

Floden, A.M., and Combs, C.K. (2007). Microglia repetitively isolated from in vitro mixed glial cultures retain their initial phenotype. J Neurosci Methods 164, 218-224. doi: 10.1016/j.jneumeth.2007.04.018

Floden, A.M., and Combs, C.K. (2011). Microglia demonstrate age-dependent interaction with amyloid-beta fibrils. J Alzheimers Dis 25, 279-293. doi: 10.3233/JAD-2011-101014

Fu, G.X., Chen, A.F., Zhong, Y., Zhao, J., and Gu, Y.J. (2016). Decreased serum level of HMGB1 and MyD88 during human aging progress in healthy individuals. Aging Clin Exp Res 28, 175-180. doi: 10.1007/s40520-015-0402-8

Fujita, K., Motoki, K., Tagawa, K., Chen, X., Hama, H., Nakajima, K., Homma, H., Tamura, T., Watanabe, H., Katsuno, M., et al. (2016). HMGB1, a pathogenic molecule that induces neurite degeneration via TLR4-MARCKS, is a potential therapeutic target for Alzheimer's disease. Sci Rep 6, 31895. doi: 10.1038/srep31895

Fuller, A.D., and Van Eldik, L.J. (2008). MFG-E8 regulates microglial phagocytosis of apoptotic neurons. J Neuroimmune Pharmacol 3, 246-256. doi: 10.1007/s11481-008-9118-2

Gao, H.M., Zhou, H., Zhang, F., Wilson, B.C., Kam, W., and Hong, J.S. (2011). HMGB1 acts on microglia Mac1 to mediate chronic neuroinflammation that drives progressive neurodegeneration. J Neurosci 31, 1081-1092. doi: 10.1523/JNEUROSCI.3732-10.2011

Gingras, M., Gagnon, V., Minotti, S., Durham, H.D., and Berthod, F. (2007). Optimized protocols for isolation of primary motor neurons, astrocytes and microglia from embryonic mouse spinal cord. J Neurosci Methods 163, 111-118. doi: 10.1016/j.jneumeth.2007.02.024

Gordo, A.C., Falcão, A.S., Fernandes, A., Brito, M.A., Silva, R.F.M., and Brites, D. (2006). Unconjugated bilirubin activates and damages microglia. J Neurosci Res 84, 194-201. doi: 10.1002/jnr.20857

Grabert, K., Michoel, T., Karavolos, M.H., Clohisey, S., Baillie, J.K., Stevens, M.P., Freeman, T.C., Summers, K.M., and Mccoll, B.W. (2016). Microglial brain region-dependent diversity and selective regional sensitivities to aging. Nat Neurosci 19, 504-516. doi: 10.1038/nn.4222

Graeber, M.B. (2010). Changing Face of Microglia. Science 330, 783-788. doi: 10.1126/science.1190929

Guedes, J.R., Custodia, C.M., Silva, R.J., De Almeida, L.P., Pedroso De Lima, M.C., and Cardoso, A.L. (2014). Early miR-155 upregulation contributes to neuroinflammation in Alzheimer's disease triple transgenic mouse model. Hum Mol Genet 23, 6286-6301. doi: 10.1093/hmg/ddu34

Guedes, J.R., Santana, I., Cunha, C., Duro, D., Almeida, M.R., Cardoso, A.M., Lima, M.C.P., and Cardoso, A.L. (2016). MicroRNA deregulation and chemotaxis and phagocytosis impairment in Alzheimer's disease. Alzheimer's & Dementia: Diagnosis, Assessment & Disease Monitoring 3, 7-17. doi: 10.1016/j.dadm.2015.11.004

Harry, G.J. (2013). Microglia during development and aging. Pharmacol Ther 139, 313-326. doi: 10.1016/j.pharmthera.2013.04.013

He, Q., You, H., Li, X.M., Liu, T.H., Wang, P., and Wang, B.E. (2012). HMGB1 promotes the synthesis of pro-IL-1beta and pro-IL-18 by activation of p38 MAPK and NF-kappaB through receptors for advanced glycation end-products in macrophages. Asian Pac J Cancer Prev 13, 1365-1370.

Heneka, M.T., Carson, M.J., El Khoury, J., Landreth, G.E., Brosseron, F., Feinstein, D.L., Jacobs, A.H., Wyss-Coray, T., Vitorica, J., Ransohoff, R.M., et al. (2015a). Neuroinflammation in Alzheimer's disease. Lancet Neurol 14, 388-405. doi: 10.1016/S1474-4422(15)70016-5

Heneka, M.T., Carson, M.J., Khoury, J.E., Landreth, G.E., Brosseron, F., Feinstein, D.L., Jacobs, A.H., Wyss-Coray, T., Vitorica, J., Ransohoff, R.M., et al. (2015b). Neuroinflammation in Alzheimer's disease. Lancet Neurol 14, 388-405. doi: 10.1016/S1474-4422(15)70016-5

Heyn, J., Luchting, B., Hinske, L.C., Hubner, M., Azad, S.C., and Kreth, S. (2016). miR-124a and miR-155 enhance differentiation of regulatory T cells in patients with neuropathic pain. J Neuroinflammation 13, 248. doi: 10.1186/s12974-016-0712-6

Hjorth, E., Zhu, M., Toro, V.C., Vedin, I., Palmblad, J., Cederholm, T., Freund-Levi, Y., Faxen-Irving, G., Wahlund, L.O., Basun, H., et al. (2013). Omega-3 fatty acids enhance phagocytosis of Alzheimer's disease-related amyloid-beta42 by human microglia and decrease inflammatory markers. J Alzheimers Dis 35, 697-713. doi: 10.3233/JAD-130131

Hoeijmakers, L., Heinen, Y., Van Dam, A.M., Lucassen, P.J., and Korosi, A. (2016). Microglial Priming and Alzheimer's Disease: A Possible Role for (Early) Immune

Challenges and Epigenetics? Front Hum Neurosci 10, 398. doi: 10.3389/fnhum.2016.00398

Hof, P.R., and Mobbs, C.V. (2009). Handbook of the neuroscience of aging. Oxford, UK: Academic Press.

Ito, S., Kimura, K., Haneda, M., Ishida, Y., Sawada, M., and Isobe, K. (2007). Induction of matrix metalloproteinases (MMP3, MMP12 and MMP13) expression in the microglia by amyloid-beta stimulation via the PI3K/Akt pathway. Exp Gerontol 42, 532-537. doi: 10.1016/j.exger.2006.11.012

Jiang, M., Xiang, Y., Wang, D., Gao, J., Liu, D., Liu, Y., Liu, S., and Zheng, D. (2012). Dysregulated expression of miR-146a contributes to age-related dysfunction of macrophages. Aging Cell 11, 29-40. doi: 10.1111/j.1474-9726.2011.00757.x

Kettenmann, H., Hanisch, U.K., Noda, M., and Verkhratsky, A. (2011). Physiology of microglia. Physiol Rev 91, 461-553. doi: 10.1152/physrev.00011.2010

Kim, H.J., Ajit, D., Peterson, T.S., Wang, Y., Camden, J.M., Gibson Wood, W., Sun, G.Y., Erb, L., Petris, M., and Weisman, G.A. (2012). Nucleotides released from Abeta(1)(-)(4)(2) -treated microglial cells increase cell migration and Abeta(1)(-)(4)(2) uptake through P2Y(2) receptor activation. J Neurochem 121, 228-238. doi: 10.1111/j.1471-4159.2012.07700.x

Klionsky, D.J., Abdelmohsen, K., Abe, A., Abedin, M.J., Abeliovich, H., Acevedo Arozena, A., Adachi, H., Adams, C.M., Adams, P.D., Adeli, K., et al. (2016). Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). Autophagy 12, 1-222. doi: 10.1080/15548627.2015.1100356

Kohman, R.A., Bhattacharya, T.K., Wojcik, E., and Rhodes, J.S. (2013). Exercise reduces activation of microglia isolated from hippocampus and brain of aged mice. J Neuroinflammation 10, 114. doi: 10.1186/1742-2094-10-114

Krabbe, G., Halle, A., Matyash, V., Rinnenthal, J.L., Eom, G.D., Bernhardt, U., Miller, K.R., Prokop, S., Kettenmann, H., and Heppner, F.L. (2013). Functional impairment of microglia coincides with Beta-amyloid deposition in mice with Alzheimer-like pathology. PLoS One 8, e60921. doi: 10.1371/journal.pone.0060921

Leovsky, C., Fabian, C., Naaldijk, Y., Jager, C., Jang, H.J., Bohme, J., Rudolph, L., and Stolzing, A. (2015). Biodistribution of in vitro-derived microglia applied intranasally and intravenously to mice: effects of aging. Cytotherapy 17, 1617-1626. doi: 10.1016/j.jcyt.2015.07.019

Li, W. (2012). Eat-me signals: keys to molecular phagocyte biology and "appetite" control. J Cell Physiol 227, 1291-1297. doi: 10.1002/jcp.22815

Li, W. (2013). Phagocyte dysfunction, tissue aging and degeneration. Ageing Res Rev 12, 1005-1012. doi: 10.1016/j.arr.2013.05.006

Li, X., Kong, D., Chen, H., Liu, S., Hu, H., Wu, T., Wang, J., Chen, W., Ning, Y., Li, Y., and Lu, Z. (2016). miR-155 acts as an anti-inflammatory factor in atherosclerosis-associated foam cell formation by repressing calcium-regulated heat stable protein 1. Sci Rep 6, 21789. doi: 10.1038/srep21789

Limatola, C., and Ransohoff, R.M. (2014). Modulating neurotoxicity through CX3CL1/CX3CR1 signaling. Front Cell Neurosci 8, 229. doi: 10.3389/fncel.2014.00229

Liu, Y., Yang, X., Guo, C., Nie, P., and Ma, J. (2013). Essential role of MFG-E8 for phagocytic properties of microglial cells. PLoS One 8, e55754. doi: 10.1371/journal.pone.0055754

Lorenzl, S., Buerger, K., Hampel, H., and Beal, M.F. (2008). Profiles of matrix metalloproteinases and their inhibitors in plasma of patients with dementia. Int Psychogeriatr 20, 67-76. doi: 10.1017/S1041610207005790

Lukiw, W.J. (2007). Micro-RNA speciation in fetal, adult and Alzheimer's disease hippocampus. Neuroreport 18, 297-300. doi: 10.1097/WNR.0b013e3280148e8b

Lull, M.E., and Block, M.L. (2010). Microglial activation and chronic neurodegeneration. Neurotherapeutics 7, 354-365. doi: 10.1016/j.nurt.2010.05.014

Lynch, A.M., Murphy, K.J., Deighan, B.F., O'reilly, J.A., Gun'ko, Y.K., Cowley, T.R., Gonzalez-Reyes, R.E., and Lynch, M.A. (2010). The impact of glial activation in the aging brain. Aging Dis 1, 262-278.

Maggio, M., Guralnik, J.M., Longo, D.L., and Ferrucci, L. (2006). Interleukin-6 in aging and chronic disease: a magnificent pathway. J Gerontol A Biol Sci Med Sci 61, 575-584. doi: 61/6/575

Mccarthy, K.D., and De Vellis, J. (1980). Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue. J Cell Biol 85, 890-902.

Merino, J.J., Muneton-Gomez, V., Alvarez, M.I., and Toledano-Diaz, A. (2016). Effects of CX3CR1 and Fractalkine Chemokines in Amyloid Beta Clearance and p-Tau Accumulation in Alzheimer,s Disease (AD) Rodent Models: Is Fractalkine a Systemic Biomarker for AD? Curr Alzheimer Res 13, 403-412.

Miller, A.M., and Stella, N. (2009). Microglial cell migration stimulated by ATP and C5a involve distinct molecular mechanisms: quantification of migration by a novel near-infrared method. Glia 57, 875-883. doi: 10.1002/glia.20813

Miners, J.S., Barua, N., Kehoe, P.G., Gill, S., and Love, S. (2011). Abeta-degrading enzymes: potential for treatment of Alzheimer disease. J Neuropathol Exp Neurol 70, 944-959. doi: 10.1097/NEN.0b013e3182345e46

Mizuno, T. (2012). The biphasic role of microglia in Alzheimer's disease. Int J Alzheimers Dis 2012, 737846. doi: 10.1155/2012/737846

Monsonego, A., Imitola, J., Zota, V., Oida, T., and Weiner, H.L. (2003). Microglia-mediated nitric oxide cytotoxicity of T cells following amyloid beta-peptide presentation to Th1 cells. J Immunol 171:2216-2224.

Morgan, D. (2009). The role of microglia in antibody-mediated clearance of amyloid-beta from the brain. CNS Neurol Disord Drug Targets 8, 7-15.

Mosher, K.I., and Wyss-Coray, T. (2014). Microglial dysfunction in brain aging and Alzheimer's disease. Biochem Pharmacol 88, 594-604. doi: 10.1016/j.bcp.2014.01.008

Moussaud, S., and Draheim, H.J. (2010). A new method to isolate microglia from adult mice and culture them for an extended period of time. J Neurosci Methods 187, 243-253. doi: 10.1016/j.jneumeth.2010.01.017

Murphy, A.J., Kraakman, M.J., Kammoun, H.L., Dragoljevic, D., Lee, M.K., Lawlor, K.E., Wentworth, J.M., Vasanthakumar, A., Gerlic, M., Whitehead, L.W., et al. (2016). IL-18 Production from the NLRP1 Inflammasome Prevents Obesity and Metabolic Syndrome. Cell Metab 23, 155-164. doi: 10.1016/j.cmet.2015.09.024

Nakajima, K., and Kohsaka, S. (2004). Microglia:Neuroprotective and Neurotrophic Cells in the Central Nervous System. Current Drug Targets - Cardiovascular & Hematological Disorders 4, 65-84. doi: 10.2174/1568006043481284

Neumann, H., Kotter, M.R., and Franklin, R.J. (2009). Debris clearance by microglia: an essential link between degeneration and regeneration. Brain 132, 288-295. doi: 10.1093/brain/awn109

Njie, E.G., Boelen, E., Stassen, F.R., Steinbusch, H.W., Borchelt, D.R., and Streit, W.J. (2012). Ex vivo cultures of microglia from young and aged rodent brain reveal age-related changes in microglial function. Neurobiol Aging 33, 195 e191-112. doi: 10.1016/j.neurobiolaging.2010.05.008

Noren Hooten, N., Abdelmohsen, K., Gorospe, M., Ejiogu, N., Zonderman, A.B., and Evans, M.K. (2010). microRNA expression patterns reveal differential expression of target genes with age. PLoS One 5, e10724. doi: 10.1371/journal.pone.0010724

Park, J.S., Svetkauskaite, D., He, Q., Kim, J.Y., Strassheim, D., Ishizaka, A., and Abraham, E. (2004). Involvement of toll-like receptors 2 and 4 in cellular activation by high mobility group box 1 protein. J Biol Chem 279, 7370-7377. doi: 10.1074/jbc.M306793200

Ponomarev, E.D., Veremeyko, T., and Weiner, H.L. (2013). MicroRNAs are universal regulators of differentiation, activation, and polarization of microglia and

macrophages in normal and diseased CNS. Glia 61, 91-103. doi: 10.1002/glia.22363

Prince, M., Bryce, R., Albanese, E., Wimo, A., Ribeiro, W., and Ferri, C.P. (2013). The global prevalence of dementia: a systematic review and metaanalysis. Alzheimers Dement 9, 63-75 e62. doi: 10.1016/j.jalz.2012.11.007

Qiu, W.Q., Ye, Z., Kholodenko, D., Seubert, P., and Selkoe, D.J. (1997). Degradation of amyloid beta-protein by a metalloprotease secreted by microglia and other neural and non-neural cells. J Biol Chem 272, 6641-6646.

Regen, F., Hellmann-Regen, J., Costantini, E., and Reale, M. (2017). Neuroinflammation and Alzheimer's Disease: Implications for Microglial Activation. Curr Alzheimer Res. doi: 10.2174/1567205014666170203141717

Rippo, M.R., Olivieri, F., Monsurro, V., Prattichizzo, F., Albertini, M.C., and Procopio, A.D. (2014). MitomiRs in human inflamm-aging: a hypothesis involving miR-181a, miR-34a and miR-146a. Exp Gerontol 56, 154-163. doi: 10.1016/j.exger.2014.03.002

Roszer, T. (2015). Understanding the Mysterious M2 Macrophage through Activation Markers and Effector Mechanisms. Mediators Inflamm 2015, 816460. doi: 10.1155/2015/816460

Rubio-Perez, J.M., and Morillas-Ruiz, J.M. (2012). A review: inflammatory process in Alzheimer's disease, role of cytokines. ScientificWorldJournal 2012, 756357. doi: 10.1100/2012/756357

Ryu, K.Y., Cho, G.S., Piao, H.Z., and Kim, W.K. (2012). Role of TGF-beta in Survival of Phagocytizing Microglia: Autocrine Suppression of TNF-alpha Production and Oxidative Stress. Exp Neurobiol 21, 151-157. doi: 10.5607/en.2012.21.4.151

Saresella, M., La Rosa, F., Piancone, F., Zoppis, M., Marventano, I., Calabrese, E., Rainone, V., Nemni, R., Mancuso, R., and Clerici, M. (2016). The NLRP3 and NLRP1 inflammasomes are activated in Alzheimer's disease. Mol Neurodegener 11, 23. doi: 10.1186/s13024-016-0088-1

Saura, J., Tusell, J.M., and Serratosa, J. (2003). High-yield isolation of murine microglia by mild trypsinization. Glia 44, 183-189. doi: 10.1002/glia.10274

Schwartz, M., Butovsky, O., Bruck, W., and Hanisch, U.K. (2006). Microglial phenotype: is the commitment reversible? Trends Neurosci 29, 68-74. doi: 10.1016/j.tins.2005.12.005

Shibata, M., Lu, T., Furuya, T., Degterev, A., Mizushima, N., Yoshimori, T., Macdonald, M., Yankner, B., and Yuan, J. (2006). Regulation of intracellular accumulation of mutant Huntingtin by Beclin 1. J Biol Chem 281, 14474-14485. doi: 10.1074/jbc.M600364200

Sierra, A., Gottfried-Blackmore, A.C., Mcewen, B.S., and Bulloch, K. (2007). Microglia derived from aging mice exhibit an altered inflammatory profile. Glia 55, 412-424. doi: 10.1002/glia.20468

Sikora, E., Arendt, T., Bennett, M., and Narita, M. (2011). Impact of cellular senescence signature on ageing research. Ageing Res Rev 10, 146-152. doi: 10.1016/j.arr.2010.10.002

Silva, S.L., Vaz, A.R., Barateiro, A., Falcão, A.S., Fernandes, A., Brito, M.A., Silva, R.F., and Brites, D. (2010). Features of bilirubin-induced reactive microglia: from phagocytosis to inflammation. Neurobiol Dis 40, 663-675. doi: 10.1016/j.nbd.2010.08.010

Sondag, C.M., Dhawan, G., and Combs, C.K. (2009). Beta amyloid oligomers and fibrils stimulate differential activation of primary microglia. J Neuroinflammation 6, 1. doi: 10.1186/1742-2094-6-1

Streit, W.J., Braak, H., Xue, Q.S., and Bechmann, I. (2009). Dystrophic (senescent) rather than activated microglial cells are associated with tau pathology and likely precede neurodegeneration in Alzheimer's disease. Acta Neuropathol 118, 475-485. doi: 10.1007/s00401-009-0556-6.

Streit, W.J., Miller, K.R., Lopes, K.O., and Njie, E. (2008). Microglial degeneration in the aging brain-bad news for neurons? Front Biosci 13, 3423-3438. doi: 2937

Sudduth, T.L., Schmitt, F.A., Nelson, P.T., and Wilcock, D.M. (2013). Neuroinflammatory phenotype in early Alzheimer's disease. Neurobiol Aging 34, 1051-1059. doi: 10.1016/j.neurobiolaging.2012.09.012

Sun, Q., Wu, W., Hu, Y.C., Li, H., Zhang, D., Li, S., Li, W., Li, W.D., Ma, B., Zhu, J.H., Zhou, M.L., and Hang, C.H. (2014). Early release of high-mobility group box 1 (HMGB1) from neurons in experimental subarachnoid hemorrhage in vivo and in vitro. J Neuroinflammation 11, 106. doi: 10.1186/1742-2094-11-106

Tambuyzer, B.R., Ponsaerts, P., and Nouwen, E.J. (2009). Microglia: gatekeepers of central nervous system immunology. J Leukoc Biol 85:352-370. doi:10.1189/jlb.0608385

Torres-Platas, S.G., Comeau, S., Rachalski, A., Bo, G.D., Cruceanu, C., Turecki, G., Giros, B., and Mechawar, N. (2014). Morphometric characterization of microglial phenotypes in human cerebral cortex. J Neuroinflammation 11, 12. doi: 10.1186/1742-2094-11-12

Varnum, M.M., and Ikezu, T. (2012). The classification of microglial activation phenotypes on neurodegeneration and regeneration in Alzheimer's disease brain. Arch Immunol Ther Exp (Warsz) 60, 251-266. doi: 10.1007/s00005-012-0181-2

Varvel, N.H., Bhaskar, K., Kounnas, M.Z., Wagner, S.L., Yang, Y., Lamb, B.T., and Herrup, K. (2009). NSAIDs prevent, but do not reverse, neuronal cell cycle reentry in a mouse model of Alzheimer disease. J Clin Invest 119, 3692-3702. doi: 10.1172/JCI39716

Von Bernhardi, R. (2010). Immunotherapy in Alzheimer's disease: where do we stand? Where should we go? J Alzheimers Dis 19, 405-421. doi: 10.3233/JAD-2010-1248

Von Bernhardi, R., Eugenin-Von Bernhardi, L., and Eugenin, J. (2015). Microglial cell dysregulation in brain aging and neurodegeneration. Front Aging Neurosci 7, 124. doi: 10.3389/fnagi.2015.00124

Walker, D.G., and Lue, L.F. (2015). Immune phenotypes of microglia in human neurodegenerative disease: challenges to detecting microglial polarization in human brains. Alzheimers Res Ther 7, 56. doi: 10.1186/s13195-015-0139-9

Wang, W.Y., Tan, M.S., Yu, J.T., and Tan, L. (2015). Role of pro-inflammatory cytokines released from microglia in Alzheimer's disease. Ann Transl Med 3, 136. doi: 10.3978/j.issn.2305-5839.2015.03.49

Wang, X.X., Tan, M.S., Yu, J.T., and Tan, L. (2014). Matrix metalloproteinases and their multiple roles in Alzheimer's disease. Biomed Res Int 2014, 908636. doi: 10.1155/2014/908636

Wilcock, D.M. (2012). A changing perspective on the role of neuroinflammation in Alzheimer's disease. Int J Alzheimers Dis 2012, 495243. doi: 10.1155/2012/495243

Wojtera, M., Sobow, T., Kloszewska, I., Liberski, P.P., Brown, D.R., and Sikorska, B. (2012). Expression of immunohistochemical markers on microglia in Creutzfeldt-Jakob disease and Alzheimer's disease: morphometric study and review of the literature. Folia Neuropathol 50, 74-84. doi: 18392

Wynne, A.M., Henry, C.J., Huang, Y., Cleland, A., and Godbout, J.P. (2010). Protracted downregulation of CX3CR1 on microglia of aged mice after lipopolysaccharide challenge. Brain Behav Immun 24, 1190-1201. doi: 10.1016/j.bbi.2010.05.011

Wyss-Coray, T. (2006). Inflammation in Alzheimer disease: driving force, bystander or beneficial response? Nat Med 12, 1005-1015. doi: 10.1038/nm1484

Xavier, A.L., Menezes, J.R., Goldman, S.A., and Nedergaard, M. (2014). Fine-tuning the central nervous system: microglial modelling of cells and synapses. Philos Trans R Soc Lond B Biol Sci 369, 20130593. doi: 10.1098/rstb.2013.0593

Zaki, M.H., Vogel, P., Body-Malapel, M., Lamkanfi, M., and Kanneganti, T.D. (2010). IL-18 production downstream of the Nlrp3 inflammasome confers protection against colorectal tumor formation. J Immunol 185, 4912-4920. doi: 10.4049/jimmunol.1002046

Zare-Shahabadi, A., Masliah, E., Johnson, G.V., and Rezaei, N. (2015). Autophagy in Alzheimer's disease. Rev Neurosci 26, 385-395. doi: 10.1515/revneuro-2014-0076

Zhang, F., Nance, E., Alnasser, Y., Kannan, R., and Kannan, S. (2016). Microglial migration and interactions with dendrimer nanoparticles are altered in the presence of neuroinflammation. J Neuroinflammation 13, 65. doi: 10.1186/s12974-016-0529-3

Chapter 4

REDUCED MICROGLIAL RESPONSIVENESS IN THE EARLY ALZHEIMER'S DISEASE STAGE OF 3xTg-AD MICE PRECEDE INFLAMMATION

Cláudia Caldeira[1], Carolina Cunha[1], Ana Rita Vaz[1,2], Elisabete Ferreiro[3], Adelaide Fernandes[1,2], Dora Brites[1,2]

[1] Research Institute for Medicines (iMed.ULisboa), Faculdade de Farmácia, Universidade de Lisboa, Lisbon, Portugal

[2] Department of Biochemistry and Human Biology, Faculdade de Farmácia, Universidade de Lisboa, Lisbon, Portugal

[3] CNC-Center for Neuroscience and Cell Biology, University of Coimbra, Coimbra, Portugal; Institute for Interdisciplinary Research, University of Coimbra (IIIUC), Polo II, Coimbra, Portugal.

(In preparation)

Acknowledgments

This work was supported by FEDER (COMPETE Programme) and by National funds (FCT - Fundação para a Ciência e a Tecnologia) Project FCT-PEst-OE/SAU/UI4013 to iMed.ULisboa and Project FCT- EXPL/NEU-NMC/1003/2013 to AF, and, in part, by the EU Joint Programme - Neurodegenerative Disease Research (JPND) MADGIC project JPco-fuND/0003/2015 to DB, as well as by the prize Edgar Cruz e Silva 2012 from Grupo de Estudo do Envelhecimento Cerebral e Demência to DB. ARV holds a postdoctoral research fellowship (SFRH/BPD/76590/2011), and C Cunha is recipient of a PhD fellowship (SFRH/BD/91316/2012), all from FCT.

Abstract

Alzheimer's disease (AD), the most common cause for age-associated dementia, has a complex nature being characterized by a spatial and temporal accumulation of amyloid-β (Aβ) peptide, neurofibrillary tangle formation, and neuronal loss. Reactive astrocytes and microglia in the vicinity of senile plaques release inflammatory mediators that contribute for the neuroinflammatory status that characterize AD pathogenesis. Microglia may have either a neuroprotective or neurotoxic function, depending on the progression stage. The current study examines the temporal profile of microglia deactivation/activation in the 3xTg-AD mouse model prior to neurofibrillary tangle detection. Samples from the cortex and hippocampus of 3-, 6- and 9-months old 3xTg-AD animals were used to determine microglia inflammatory specific markers. Here, we observed an intriguing CD11b downregulation at the 3-months 3xTg-AD animals, both in cortex and hippocampus, suggesting the existence of an inhibitory niche at this early AD stage. This finding revealed to also be associated with a depressed expression profile of inflammatory cytokines (e.g. TNF-α, IL-1β, IL-6, IL-18 and HMGB1) and, although the cause for such inhibition is unknown, may reflect a protective role against neurodegeneration. However, overexpression of some of these inflammatory mediators were detected either the hippocampus or the cortex, or both, at 9-months of age. Coincident with the current results, differential M1 (e.g. MHC class II and CEBP-α) and M2 (e.g. SOCS1, TGF-β and Arginase 1) phenotypic markers were obtained between the earlier and later stages of neuropathogenesis, if we consider their low expression at 3-months, together with their upregulation (e.g. iNOS, SOCS1 and Arginase 1) at 9-months. Contrasting with these findings, inflammatory microRNAs (inflamma-miRs) associated to these polarized microglia phenotypes (e.g. miR-124, miR-155 and miR-146a) were found elevated at the 3-months animals. Interestingly, only miR-155 maintained a temporal upregulation in both hippocampus and cortex, sustaining its potential as a predictive AD biomarker. We next searched candidate target genes of miR-155 and identified new immune-related molecules that were mostly underexpressed in the 3xTgAD animals by comparison with the wild type mice, both at 3-months and 9-months of age. Overall, our data clearly indicate a dysfunctional immune response of 3xTgAD animals when compared to aged-matched WT. While inhibition of microglial function is a feature of the early neuropathogenesis towards neuroprotection, a dysfunctional inflammatory status is later observed. These findings corroborate the existence of diverse pathological stages along AD progression, with distinct microglia phenotypes, reinforcing the need of different disease-modifying therapies.

Keywords: Alzheimer's disease, microglia reactivity, 3xTg-AD animal model, inflamma-microRNAs, miR-155 targets

1. Introduction

Alzheimer's disease (AD) is nowadays the most common cause of dementia in elderly people, without any effective treatment. Therefore, it is extremely important to understand the causes of Alzheimer's and make an easy diagnosis as early as possible. To address this issue, animal models become essential to further understand the molecular and cellular markers that trigger the onset of AD-related cognitive decline, as well as to perform preclinical testing of novel therapeutics. Several mouse models have been generated in an effort to express similar behavior and neuropathology to that observed in human AD patients, although it is now believed that no model can completely mimic AD. To date, most used animal models are transgenic mice that overexpress human genes associated with familial AD, leading to the formation of senile plaques of peptide amyloid-β (Aβ) [e.g. overexpression of human amyloid precursor protein (*APP*) alone or with Presenilin 1 (*PS1*)] and neurofibrillary tangles of hyperphosphorylated tau protein (e.g. overexpression of human *MAPT*), two hallmarks of AD (Drummond and Wisniewski, 2017). Recently, the validity of such models has been questioned by the high failure rate of clinical trials of AD therapeutics (>99%) that were successful in preclinical trials using these models (Cummings et al., 2014). Nevertheless, the data generated by experimental AD animal models can bring new highlights about specific aspects of AD pathogenesis if researchers take into account the limitations of each model.

Although the introduction of wild-type forms of APP and PS1, as found in normal and non-familial AD cases, does not elicit AD pathology in mice, the transgenic mice overexpressing pathological human mutant proteins easily show Aβ aggregation, cognitive deficits and some abnormal tau-phosphorylation (Duyckaerts et al., 2008; Howlett and Richardson, 2009; Morrissette et al., 2009). The mice model APP/PS1δE9 that express APP with the Swedish mutation and mutant human PS1 with a deletion of exon 9 show formation of amyloid plaques prior to typical cognitive impairments (Jankowsky et al., 2004; Volianskis et al., 2010), being used to examine pathophysiological events associated to preclinical AD (Zou et al., 2016). Another useful model of intraneuronal Aβ-induced neurodegeneration is the 5XFAD (Eimer and Vassar, 2013). This mouse model expresses five familiar AD mutations, two for PS1 and three for APP, that induces Aβ overproduction, to accelerate plaque development (Oakley et al., 2006). Consequently, 5XFAD mice develop intraneuronal Aβ at 1.5-months of age, amyloid plaques at 2-months, and significant neuron loss at 9-months (Oakley et al., 2006). However, these models do not exhibit the development of neurofibrillary tangles, as well as massive neuronal loss (Schwab et al., 2009). On the other hand, the triple-

transgenic mice model (3xTg-AD) progressively develops both Aβ plaques and neurofibrillary tangles with a temporal and spatial distribution that recapitulates the disease in humans (Oddo et al., 2003a; Oddo et al., 2003b). These animals express three major genes associated with AD: APP_{Swe}, $PS1_{M146V}$, and tau_{P301L}, leading to intraneuronal Aβ accumulation first in cortical region that later spreads into hippocampus, followed by the emergence of neurofibrillary tangles in hippocampus that then spread to cortical regions (Oddo et al., 2003a; Oddo et al., 2003b). In these animals cognitive decline is detected as early as 4-months, when intraneuronal Aβ accumulates in cortex, hippocampus and also in amygdala (Billings et al., 2005), which is consistent with the amyloid cascade hypothesis. Interestingly, the 3xTg-AD animals also show extensive astrogliosis and microgliosis with upregulation of inflammatory molecules after 6-months (Janelsins et al., 2008; Caruso et al., 2013), which is prior to neurofibrillary tangle detection.

An elevated number of activated astrocytes and microglia are usually found close to neurons and Aβ plaques (Rubio-Perez and Morillas-Ruiz, 2012). Microglia, the resident immunocompetent and phagocytic cells in the central nervous system (CNS) (Krause and Muller, 2010), can be either neuroprotective or neurotoxic, which may promote a decrease or worsening of disease progression (Schwartz et al., 2006). In fact, while microglia phagocytic capacity contributes to CNS neuroprotection from excessive Aβ, microglial activation by Aβ soluble oligomers promotes excitotoxicity and neurodegeneration by the release of several inflammatory cytokines, such as tumour necrosis factor (TNF)-α and interleukin (IL)-1β, contributing to onset and progression of AD (Mizuno, 2012; Lopategui Cabezas et al., 2014). This M1/pro-inflammatory microglia phenotype has been widely reported in AD studies, however upregulation of the M2/resolution of damage marker Arginase 1 (Colton et al., 2006) and co-expression of M1, M2a, M2b and M2c markers were also detected in AD cases (Wilcock, 2012; Sudduth et al., 2013). In addition, a dystrophic microglia phenotype was also identified in AD patient autopsied samples (Bachstetter et al., 2015).

We have previously addressed changes in microglia reactivity with age and Aβ challenge using an in vitro aging model developed in our laboratory (Caldeira et al., 2014). We showed that several neuroprotective functions, including phagocytosis, migration abilities, and autophagy are impaired by *in vitro* ageing (Caldeira et al., 2014), while Aβ-induced inflammatory response is also reduced in aged cells with compromised expression of inflamma-miRs (Caldeira et al., 2017). Most attractively we demonstrated that exposure of young/reactive microglia to Aβ promote cellular senescence (Caldeira et al., 2017), in accordance with the link between AD and immunosenescence (Richartz et al., 2005).

In the present study we decided to further evaluate the microglia response and different phenotype populations in the animal model of AD that develops both amyloid-β and Tau pathology (Oddo et al., 2003a) in parallel with inflammation and microglia activation (Janelsins et al., 2005), the 3xTg-AD. Based on our previous studies and in recent literature we hypothesized that microglia may present differential populations along disease progression in the *in vivo* model. Hence, our main interest was to determine microglia specific markers in the cortex and hippocampus of 3-, 6- and 9-months old 3xTg-AD animals. For that, we evaluated the mRNA expression of specific markers of microglia reactivity, namely CD11b, inflammatory cytokines, M1 and M2-associated molecules and inflamma-miRs.

Our results surprisingly showed that CD11b was markedly reduced at 3-months both in cortex and hippocampus, suggesting reduced microglia reactivity at this earlier time-point. This response profile was also observed for the expression of inflammatory cytokines (e.g. TNF-α, IL-1β, IL-6, IL-18 and HMGB1), as well as for both M1 (e.g. MHC class II and CEBP-α) and M2 (e.g. SOCS1, TGF-β and Arginase 1) phenotypic markers, followed by later increase of specific markers. Interestingly, inflamma-miRs associated with microglia polarization was all elevated at 3-months animals, but only miR-155 kept elevated at 9-months animals, suggesting a potential role as an early AD biomarker. MiR-155 target analysis further detected new immune-related molecules that are differently expressed in WT vs. 3xTg-AD animals both at early (3-months) and more advanced (9-months) stages of AD. Collectively, we may hypothesize that the 3xTg-AD animals show a dysfunctional immune response when compared to aged-matched WT, showing first a constrained microglia response, possibly to hold damage, followed by an inflammatory response in parallel with failure of immune neuroprotective role.

2. Materials and Methods

2.1. Animals

Animal care followed the recommendations of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Council Directive 86/609/EEC) and National Law 1005/92 (rules for protection of experimental animals). All animal procedures were approved by the Institutional animal care and use committee. Every effort was made to minimize suffering and the number of animals used in this study.

The triple-transgenic animal model of Alzheimer's disease, 3xTg-AD mice, have three mutations associated with AD, namely human amyloid precursor protein Swedish (APP_{Swe}), human presenilin-1 M146V ($PS1_{M146V}$), and the P301L mutation of human tau

(tau_{P301L}), allowing the development of amyloid plaques, acceleration of the deposition rate, and neurofibrillary tangle pathology, respectively (Oddo et al., 2003b). The 3xTg-AD mice was supplied from a colony implemented in the animal house facilities of the Center for Neuroscience and Cell Biology, University of Coimbra, Portugal, obtained from Dr Frank LaFerla laboratory at the Department of Neurobiology and Behavior and Institute for Brain Aging and Dementia, University of California at Irvine. The animals were found to have the same phenotypic and behavioral characteristics, as previously described by Dr Frank LaFerla group (Oddo et al., 2003a; Oddo et al., 2003b). The animals were maintained under controlled light and environmental conditions (12 h dark/light cycle, 23 ± 1ºC, 55 ± 5% relative humidity), having access to food and water. Age-matched non-transgenic/wild-type (WT) animals were used as controls. The animals were killed at 3- or 9-months of age and the brains were removed following transcardial perfusion with 20 ml of an ice-cold 0.9% NaCl solution. The brain hemispheres were separated and used for protein and mRNA extraction. For this purpose, the hemisphere was placed on an acrylic matrix and a 4 mm coronal section was cut with a stainless steel razor. The hippocampal and cortical regions from this section were dissected and kept at -80 ºC until protein or mRNA extraction.

2.2. Evaluation of APP/Aβ expression

Detection of APP/Aβ protein expression was processed by Western Blot as usual in our laboratory (Barateiro et al., 2012). Total protein extracts were obtained from brain extracts collected from the cortex and hippocampus of 3xTg-AD mice and their WT littermates. Briefly, tissues samples were lysed using TRIzol® (LifeTechnologies), according to manufacturer's instructions. Protein extracts were obtained as previously described (Simões et al., 2013) with minor alterations, and stored at -80ºC. Protein concentrations were determined using BioRad protein assay (Bio-Rad, CA, USA). Cell extracts containing equal amounts of protein (100-150 µg) were separated on a 10 to 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. The membranes were blocked with 5% non-fat milk, incubated with the primary antibody mouse anti-Aβ (6E10) antibody (1:200, BioLegend), that detects both APP and Aβ species, and mouse β-actin (1:5,000; Sigma-Aldrich) overnight at 4ºC, and then with horseradish peroxidase-labeled secondary antibodies for 1 h at room temperature. After extensive washes with saline buffer, immunoreactive bands were detected by LumiGLO® (Cell Signaling, Beverly, MA, USA) and visualized by chemiluminescence with ChemiDoc (Bio-Rad, CA, USA). Expression was quantified by computerized image analysis using the Quantity One 1-D Analysis

Software (Bio-Rad, CA, USA). Results were normalized to β-actin expression and expressed as fold *vs.* 3-month cortex WT.

2.3. Gene and microRNA expression profiling

Determination of mRNA expression was performed by RealTime PCR (RT-PCR) as usual in our laboratory (Barateiro et al., 2013). Total RNA was extracted from hippocampal and cortical tissue of 3xTg-AD and WT animals using TRIzol® (LifeTechnologies), according to manufacturer's instructions. Total RNA was quantified using Nanodrop ND-100 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Aliquots of 1 µg of total RNA were treated with DNase I and then reverse transcribed into cDNA using oligo-dT primers and SuperScript II Reverse Transcriptase under the recommended conditions. Quantitative RT-PCR (qRT-PCR) was performed using β-actin as an endogenous control to normalize the expression level of transcription factors. The sequences used as primers are represented in **Table 4.1**. qRT-PCR was performed on a RT-PCR detection (Applied Biosystems 7300 Fast Real-Time PCR System, Applied Biosystems, Madrid, Spain) using a SensiFAST SYBR® High-ROX kit (Bioline). The PCR was performed in 96-well plates with each sample performed in triplicate, and no-template control was included for each amplification product. qRT-PCR was performed under optimized conditions: 50ºC for 2 min followed by 95ºC for 2 min and finally 40 cycles at 95ºC for 0.05 min and 62ºC for 0.30 min. In order to verify the specificity of the amplification, a melt-curve analysis was performed, immediately after the amplification protocol. Non-specific products of PCR were not found in any case. The results were normalized to β-actin in the same sample and relative mRNA concentrations calculated by the formula 2-ΔΔCT , taking into account 100% efficiency for each genes. ΔCT is the value obtained, for each sample, by performing the difference between the mean CT value of each gene and the mean CT value of β-actin. ΔΔCT of one sample is the difference between its ΔCT value and ΔCT of the sample chosen as reference, in our case the 3-month cortex of WT mice.

For microRNA (miRNA) analysis, cDNA conversion was performed with the universal cDNA Synthesis Kit (Exiqon) (Cardoso et al., 2012), using 5 ng total RNA according to the following protocol: 60 min at 42ºC followed by heat-inactivation of the reverse transcriptase for 5 min at 95ºC. qRT-PCR was performed in an Biosystems 7300 Fast Real-Time PCR System (Applied Biossystems) using 96-well plates. For miRNA quantification the miRCURY LNA™ Universal RT microRNA PCR system (Exiqon) was used in combination with pre-designed primers (Exiqon) for miR-155, miR-124, miR-146a and SNORD110 (reference gene) (**Table 4.2**). The reaction conditions consisted of polymerase activation/denaturation and well-factor determination at 95ºC for 10 min,

followed by 50 amplification cycles at 95ºC for 10 s and 60ºC for 1 min (ramp-rate 1.6º/s). The miRNA fold change with respect to 3-month cortex WT was determined by the Phaffl method, taking into consideration different amplification efficiencies of miRNAs in all experiments. The amplification efficiency for each target or reference RNA was determined according to the formula: $E = 10^{(-1/S)} - 1$, where S is the slope of the obtained standard curve.

Table 4. 1 – Primer sequence used for gene expression

Gene	Primer	Sequence
Arginase1	Sense	5'-CTTGGCTTGCTTCGGAACTC-3'
	Anti-sense	5'-GGAGAAGGCGTTTGCTTAGTTC-3'
β-actin	Sense	5'-GCTCCGGCATGTGCAA-3'
	Anti-sense	5'-AGGATCTTCATGAGGTAGT-3'
CD11b	Sense	5'-CAGATCAACAATGTGACCGTATGGG-3'
	Anti-sense	5'-CATCATGTCCTTGTACTGCCGCTTG-3'
CEBP-α	Sense	5'-AGCTTACAACAGGCCAGGTTTC-3'
	Anti-sense	5'-CGGCTGGCGACATACAGTAC-3'
HMGB1	Sense	5'-CTCAGAGAGGTGGAAGACCATGT-3'
	Anti-sense	5'-GGGATGTAGGTTTTCATTTCTCTTTC-3'
IL-1β	Sense	5'-CAGGCTCCGAGATGAACAAC-3'
	Anti-sense	5'-GGTGGAGAGCTTTCAGCTCATA -3'
IL-18	Sense	5'-TGGTTCCATGCTTTCTGGACTCCT-3'
	Anti-sense	5'-TTCCTGGGCCAAGAGGAAGTG-3'
iNOS	Sense	5'-ACCCACATCTGGCAGAATGAG-3'
	Anti-sense	5'-AGCCATGACCTTTCGCATTAG-3'
MHC class II	Sense	5'-TGGGCACCATCTTCATCATTC-3'
	Anti-sense	5'-GGTCACCCAGCACACCACTT-3'
SOCS1	Sense	5'-CACCTTCTTGGTGCGCG-3'
	Anti-sense	5'-AAGCCATCTTCACGCTGAGC-3'
TGF-β	Sense	5'-CAGAGCTGCGCTTGCAGAG-3'
	Anti-sense	5'-GTCAGCAGCCGGTTACCAAG-3'
TNF-α	Sense	5'-TACTGAACTTCGGGGTGATTGGTCC -3'
	Anti-sense	5'-CAGCCTTGTCCCTTGAAGAGAACC -3'

Table 4. 2 – Primer sequences used for microRNA expression

miRs	Target Sequence (5'-3')
miR-124-3p	UAAGGCACGCGGUGAAUGCC
miR-146a-5p	UGAGAACUGAAUUCCAUGGGUU
miR-155-5p	UUAAUGCUAAUUGUGAUAGGGGU

2.4. Assessement of miR-155 target expression

To assess the expression of genes regulated by mir-155, total RNA was extracted from cortical tissue of 3xTg-AD and WT animals and quantified as described above. 500 ng of total RNA of each animal were pooled and mixed according to age (3- and 9-months) and disease condition (WT *vs.* 3xTgAD). Aliquots of 500 ng of total mixed RNA were reverse transcribed into cDNA using the RT2 First Strand Kit (Qiagen, Hilden,

Germany), according to manufacturer's instructions. qRT-PCR was performed on a RT-PCR detection (QuantStudio™ 7 Flex Real-Time PCR System, Applied Biosystems, Madrid, Spain) using the RT2 Profiler PCR Array 384-Well (4 x 96) Format (Qiagen, Hilden, Germany), and a RT2 SYBR® Green qPCR Mastermix (Qiagen, Hilden, Germany). The PCR was performed in 384-well plates, and reverse transcription control (RTC) and positive PCR control (PPC) was included to determine the reverse transcription efficiency and PCR reaction, respectively. The results were normalized to a standard set of reference genes. The threshold cycle (CT) values were analyzed with the RT2 Profiler PCR Array Data Analysis Webportal, and geometrically averaged and used for ΔΔCT calculations. Fold change was calculated by using ΔΔCT method (Livak and Schmittgen, 2001), corresponding to the ratio of gene expression between the reference or Control Group (WT) and Test Group. Fold regulation was used to better read and interpret data. Fold change values greater than 1, the fold regulation and fold change values are the same and indicate upregulated or increased gene expression. Fold change values less than 1 implies that fold-regulation is the negative inverse of the fold change, and indicate downregulated or decreased gene expression. The results were presented in a heat map and further detailed in scatter plot that compares the normalized expression of each gene between two groups (WT *vs.* 3xTgAD). Gene-specific $2^{-\Delta C_T}$ value in reference group was plotted on one axis against the corresponding value in test group on the other axis on a log base 10 scale to observe gene expression changes. Boundary lines were used to allow better visualization of upregulated and downregulated genes above and below a selected fold change value. The central line indicates unchanged gene expression. The section of the scatter plot above the fold change boundary lines contains genes upregulated in the y-axis group as compared to the x-axis group, and the section of the scatter plot below the fold change boundary lines contains genes downregulated in the y-axis group as compared to the x-axis group.

2.5. Statistical Analysis

Results of at least four different animals per experimental group are expressed as mean ± SEM. Significant differences between the parameters evaluated were determined by the two-tailed Student's *t*-test performed on the basis of equal and unequal variance, as appropriate, using GraphPad Prism® 5.0 (GraphPad Software Inc., San Diego, CA, USA). *p* value less than 0.05 were considered statistically significant.

3. Results

3.1. The 3xTg-AD animals express APP from 3-months forward

The 3xTg-AD mouse model, developed in LaFerla's laboratory, present cortical intraneuronal Aβ as early as 3-months, followed by cortical extracellular deposits and hippocampal intraneuronal Aβ at 6-months, and diffuse plaque formation at 15-months (Oddo et al., 2003a). So, first we decided to evaluate APP/Aβ protein expression in 3-, 6- and 9-month animals before further analysis to be sure that 3xTg-AD expressed increased levels of these proteins. Using the Aβ (6E10) antibody that detects both APP and Aβ we could only detect the APP species as shown in **Figure 4.1**. As expected, we observed a marked increase of APP expression in 3-months 3xTg-AD, when compared to age-matched WT animals that was maintained, although with lower magnitude in 6- and 9-months animals. Since Tau phosphorylation was only described in these 3xTg-AD at 12-months (Oddo et al., 2003a), a posterior time-point than the ones we evaluated, we have not assessed this protein.

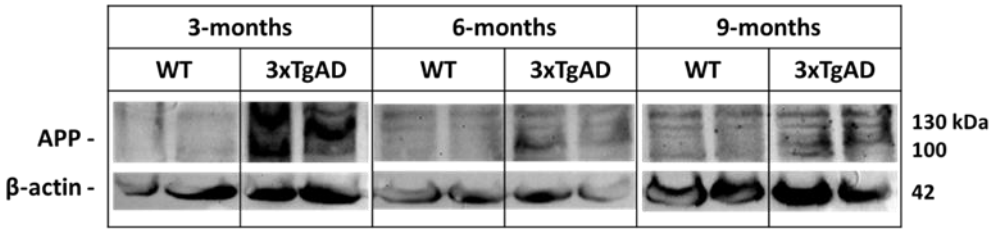

Figure 4. 1 - APP expression is increased in the 3xTg-AD animal model. Cortical samples from wild-type (WT) and 3xTg-AD animals were collected at 3-, 6- and 9-months and analyzed for APP expression by Western Blot using the Aβ (6E10) antibody. Representative bands of *n* = 2 animals per experimental group are shown.

3.2. Microglia activation is decreased at early-AD stage in the 3xTg-AD animal model

Our previous in vitro studies showed that microglia aged *in vitro* became irresponsive even when challenged with $A\beta_{1-42}$ oligomers and fibrils (Caldeira et al., 2014; Caldeira et al., 2017). Since microglia was described to behave differently along AD progression in different animal models of the disease directed to Aβ pathology (Landel et al., 2014; Martin et al., 2017), our main interest was to address the temporal expression of microglia specific markers in the 3xTg-AD animal. First, we evaluated CD11b expression in 3-, 6- and 9-months 3xTg-AD animals and aged-matched WT. We observed a marked reduction of CD11b expression in 3-months 3xTg-AD animals in both cortical and hippocampal tissues (~55% and ~65%, respectively, $p < 0.01$, **Figure 4.2**), that was lost in older animals. These results corroborate prior findings showing a slight decrease of F4/80-positive microglia in the hippocampus and entorhinal cortex of the 3xTg-AD animals (Janelsins et al., 2005). These surprising results suggest that there

may be an initial constrain of microglia response in very early stages of AD, possibly to hold an inflammatory response to the initial increase of Aβ expression already observed at 3-months as described above.

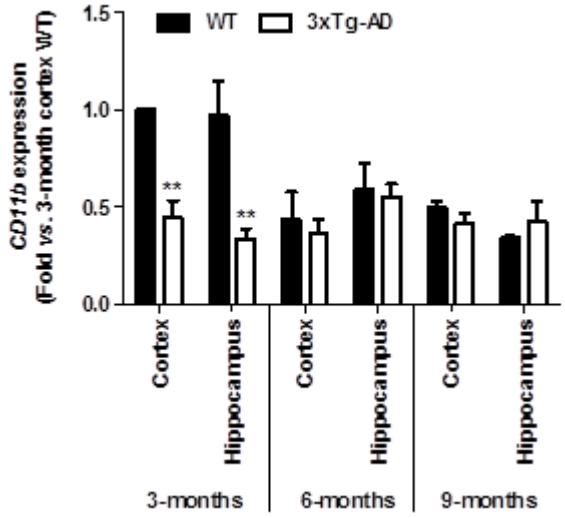

Figure 4. 2 - Microglia activation is decreased at 3-months in the 3xTg-AD animal model. Cortical and hippocampal samples from wild-type (WT) and 3xTg-AD (Tg) animals were collected at 3-, 6- and 9-months and analyzed for *CD11b* mRNA by qRT-PCR. Results are expressed as mean ± SEM, representative of n = 4 animals per experimental group and expressed as fold change *vs.* 3-month cortex WT mice. *t*- test $^{**}p <$ 0.01 *vs.* respective WT.

3.3. Microglia Inflammatory response is decreased at early-AD stage in the 3xTg-AD animal model

Microglia is usually described to be activated in early AD stages of human patients and associated with the release of pro-inflammatory cytokines (Sudduth et al., 2013). Nevertheless, only a few studies using AD animal models have looked at early months and in those cases pro-inflammatory cytokine expression is usually similar to WT animals (Martin et al., 2017) or even reduced (Guedes et al., 2014; Iwahara et al., 2017). First, we looked at cytokines that we previously showed to be released by microglia when exposed to $A\beta_{1-42}$ oligomers and fibrils (Caldeira et al., 2017). Although we first assessed animal samples from 3-, 6- and 9-months, as described above, the effects on 3- and 9-months were more conclusive, reason why we only present the results obtained for these end-points. As indicated in **Figure 4.3**, all the evaluated cytokines show a reduced mRNA expression in 3-month 3xTg-AD when compared to WT animals, but some are elevated at 9-months. Indeed, TNF-α, IL- 6, IL-1β and IL-18 expression is decreased in the cortex of 3-months 3xTg-AD by more than 40% ($p < 0.05$), while TNF-α, IL-18 and HMGB1 is also reduced in the hippocampus of these animals ($p < 0.05$). Interestingly, TNF-α and IL-6 were still reduced in the cortex of 9-month 3xTg-AD but increased in the

hippocampal area (2- and 1.5-fold, respectively, $p < 0.01$). On the other hand, IL-1β increased in the hippocampus (2.4-fold, $p < 0.01$), IL-18 in the cortex (1.4-fold, $p < 0.01$) and HMGB1 both in the cortex and hippocampus (1.3- and 2.1-fold, respectively, $p < 0.05$) of these older transgenic animals. These results, corroborate the previous findings on IL-6, TNF-α and IL-1β hippocampal reduction observed at 3-months in the same AD model (Janelsins et al., 2005; Guedes et al., 2014), and show for the first time the marked reduction of inflammatory molecules for very early stages of disease which is in accordance with the lower CD11b expression demonstrated above.

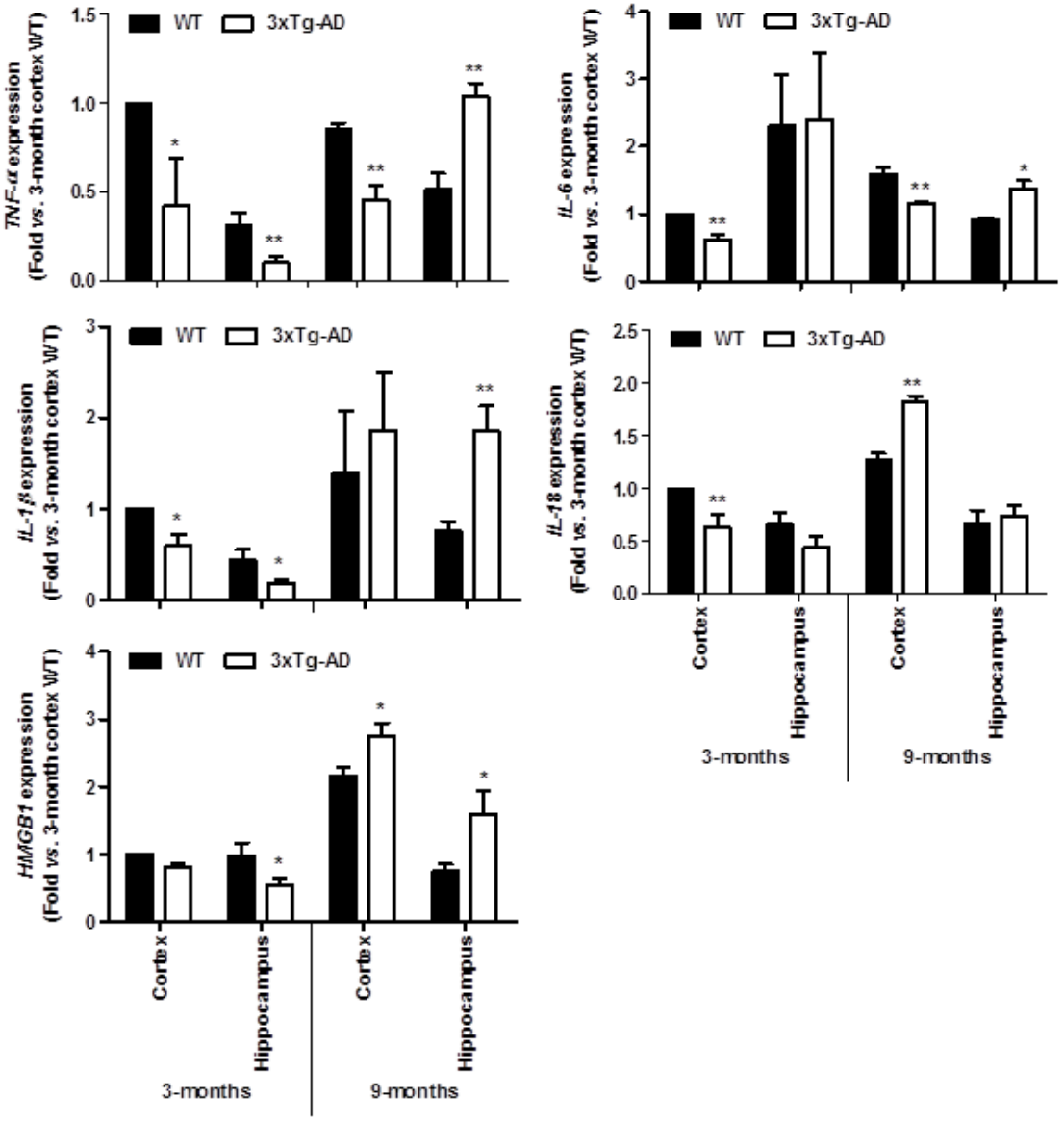

Figure 4. 3- Pro-inflammatory cytokines are reduced at 3-months in the 3xTg-AD animal model. Brain samples from wild-type (WT) and 3xTg-AD animals were collected at 3-months and analyzed for **(A)** *TNF-α*, **(B)** *IL-6*, **(C)** *IL-1β*, **(D)** *IL-18* and **(E)** *HMGB1* mRNA expression by qRT-PCR. Results are expressed in graph bars as mean ± SEM, representative of $n = 4$ animals per experimental group and expressed as fold change *vs.* 3-month cortex WT mice. *t*- test $^{*}p < 0.05$, $^{**}p < 0.01$ *vs.* respective WT.

3.4. Microglia M1 and M2 phenotype markers are decreased at early-AD stage in the 3XTg-AD animal model

Most cytokines were reduced at 3-months in the 3xTg-AD animals but further increased either at the cortex or hippocampus of 9-month transgenic animals, suggesting a first loss of microglia M1 polarization in early periods of the disorder followed by increased M1 reactivity. This later effect was also demonstrated in other AD models such as the TgAPP/PS1, the TgAPP/PS1dE9 and 5xFAD (Landel et al., 2014; Martin et al., 2017). Therefore, we further decided to characterize microglia polarization for both 3- and 9-months animals. For that we evaluated the mRNA expression of MHC class II, iNOS and CEBP-α, which are considered M1/pro-inflammatory microglia markers, and SOCS1, TGF-β and Arginase 1 as M2/anti-inflammatory microglia markers.

MHC class II is associated with microglial participation in the adaptive immune response, though it is debatable whether it is a marker of amoeboid/activated or ramified/quiescent microglia (Walker and Lue, 2015), being also associated with M2b polarized macrophages (Roszer, 2015). Here, we observed that MHC class II is reduced by ~60% at 3-months both in cortex and hippocampus ($p < 0.05$), and was still reduced at 9-months in the hippocampus (~60%, $p < 0.05$, **Figure 4A**). Curiously, also in the APP/PS1dE9 model there were no changes in microglia MHC class II along disease progression (Martin et al., 2017). CEBP-α has been described to regulate the transcription of the MHC class II gene in murine microglia (Ponomarev et al., 2011b). In accordance, we also found a decreased expression of CEBP-α in the cortex and hippocampus of 3-months 3xTg-AD animals (>35%, $p < 0.05$), but no changes were observed at 9-months (**Figure 4B**). The expression of iNOS is mostly involved in innate immune response of classic activated microglia by producing NO, which has cytotoxic effects (Colton, 2009). Although there is a tendency for reduced iNOS expression in 3-months 3xTg-AD it did not reached significance. Nevertheless, a marked increase of iNOS expression was observed at 9-months, namely in the hippocampus (~2.9-fold, $p < 0.05$, **Figure 4C**). In accordance, it was shown a microglia increase of iNOS expression also in the APP/PS1 and APP/PS1dE9 models but only after 10-15-months (Martin et al., 2017).

It has been reported that SOCS1 is involved in the shift of macrophages from M1 to M2 state (Whyte et al., 2011) and was shown to attenuate Aβ-Induced Inflammation in a microglia murine cell line (Cai et al., 2016). Once again we saw a marked reduction of SOCS1 in the cortex of 3-month 3xTg-AD animals (~50%, $p < 0.05$) that was sustained after 9-months (~25%, $p < 0.05$), but increased in the hippocampus only at 9-months (~1.8-fold, $p < 0.05$, **Figure 5A**). This pattern was also observed for Arginase 1 expression (~40%, $p < 0.05$, cortex at 3-months; and ~2.2-fold, $p < 0.05$, hippocampus

at 9-months, **Figure 5B**), a typical marker of alternative activated and deactivated microglia (Walker and Lue, 2015). Concerning TGF-β, suggested to be increased in the M2 phenotype (Roszer, 2015), was only reduced in the cortex of 3-month 3xTg-AD animals (~70%, $p < 0.05$, **Figure 5C**) with no further alteration.

Overall, both microglia M1 and M2 phenotypes are reduced in the 3xTg-AD mice in early stages when there is single cortical intraneuronal Aβ, increasing afterwards only concerning a directed innate immune response and a specific deactivated polarization.

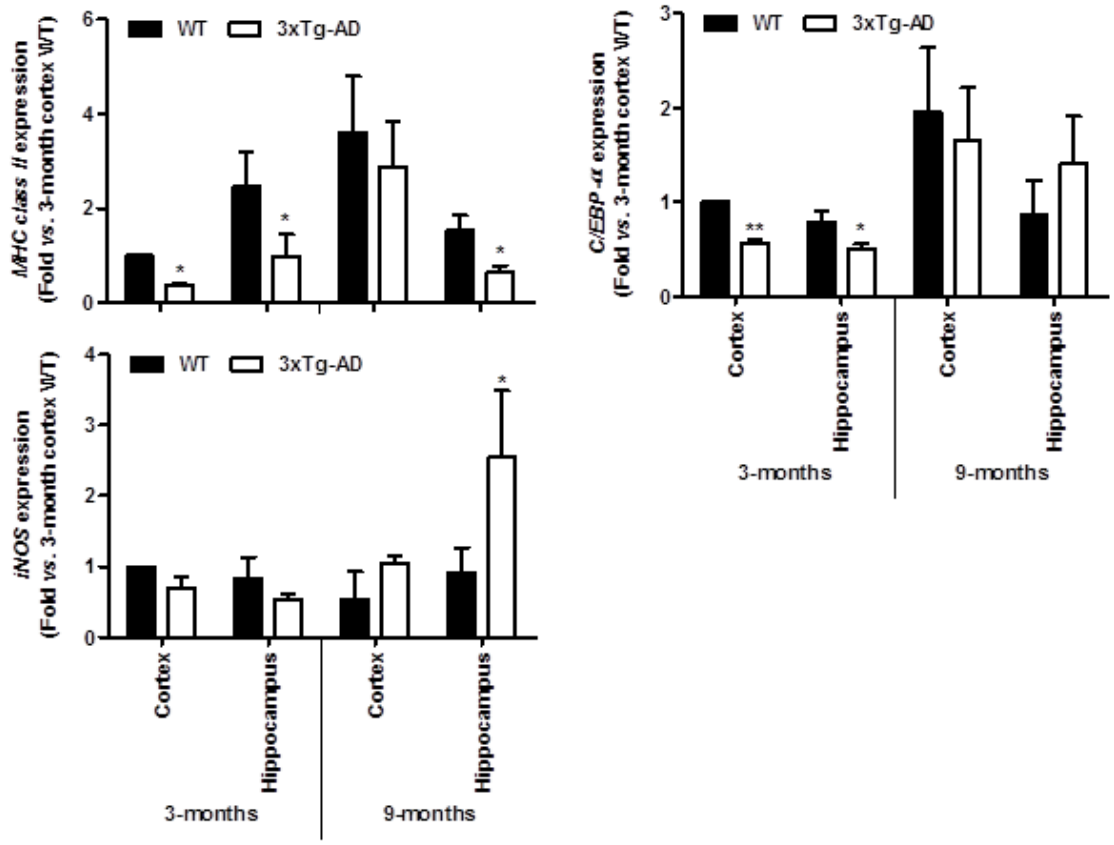

Figure 4. 4 - Microglia M1 pro-inflammatory markers are downregulated at 3-months in the 3xTg-AD animal model. Brain samples from wild-type (WT) and 3xTg-AD animals were collected at 3-months and analyzed for **(A)** *MHC class II*, **(B)** *C/EBP-α* and **(C)** *iNOS* mRNA expression by qRT-PCR. Results are expressed in graph bars as mean ± SEM, representative of $n = 4$ animals per experimental group and expressed as fold change *vs.* 3-month cortex WT mice. *t*-test $^{*}p < 0.05$ *vs.* respective WT.

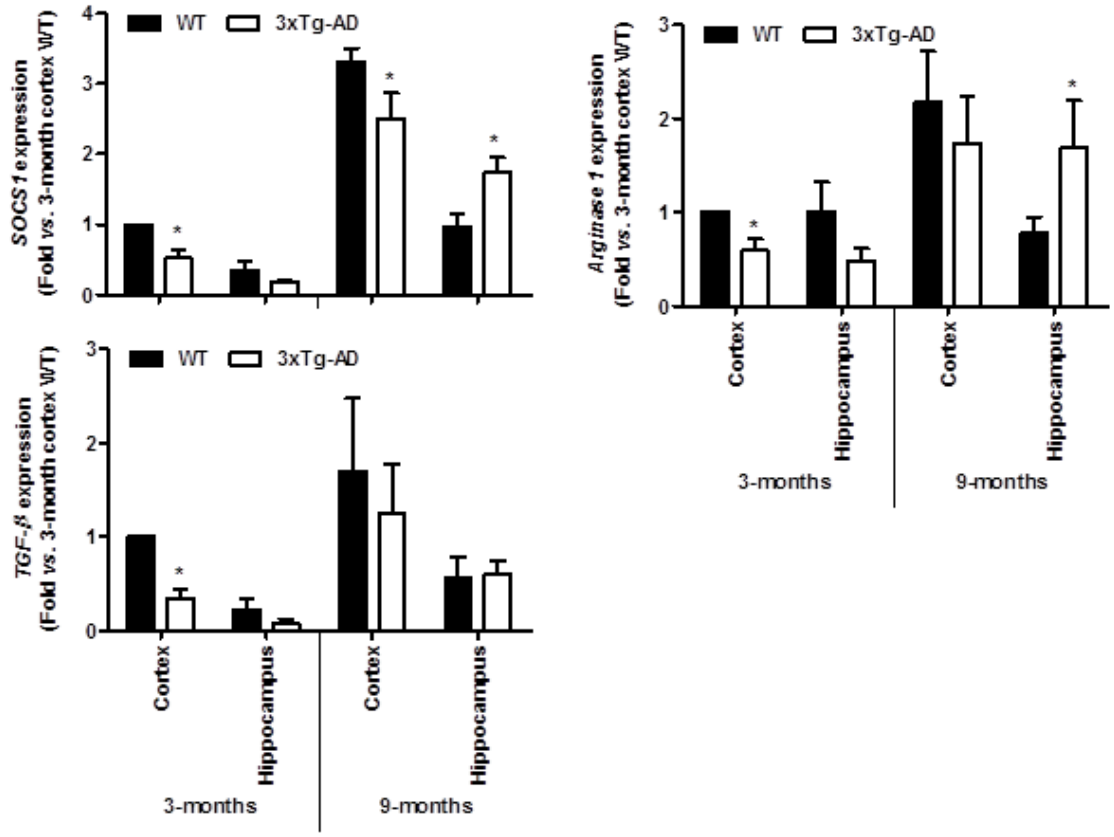

Figure 4. 5 - Microglia M2 anti-inflammatory/damage resolution markers are decreased at 3-months in the 3xTg-AD animal model. Brain samples from wild-type (WT) and 3xTg-AD animals were collected at 3-months and analyzed for **(A)** *SOCS1*, **(B)** *Arginase 1* and **(C)** *TGF-β* mRNA expression by qRT-PCR. Results are expressed in graph bars as mean ± SEM, representative of $n = 4$ animals per experimental group and expressed as fold change *vs.* 3-month cortex WT mice. *t*-test $^{*}p < 0.05$, $^{**}p < 0.01$ *vs.* respective WT.

3.5. Inflamma-miRs are elevated at early-AD stage in the 3XTG-AD animal model but only the miR-155 maintain this elevation at 9-months

Inflamma-miRs including the miR-124, miR-155 and miR-146a are known to play a fundamental role in the regulation of microglial polarization by targeting specific molecules involved in key signaling pathways (Ponomarev et al., 2013). Since we observed, a marked downregulation of M1 and M2 genes in 3-months 3xTg-AD animals, it became important to evaluate whether also these miRs could be influencing such results. Moreover, miR-155 and miR-146a were reported to be upregulated in samples from AD patients (Alexandrov et al., 2012), while miR-124 were shown to be downregulated (Wang et al., 2011), suggesting once more that changes at this epigenetic level could also be occurring in the 3xTg-AD mice model.

The miR-155 is considered a pro-inflammatory miRNA, as its expression in murine microglia cell line is upregulated in response to LPS (Cunha et al., 2016), and its

overexpression directly inhibit the expression of SOCS1 (Cardoso et al., 2012). Here, we observed a ~4-fold increase in the cortex of 3-month 3xTgTg-AD animals ($p < 0.01$) which was sustained at 9-months for both cortical and hippocampal areas (~2-fold, $p < 0.05$, **Figure 6A**). These results corroborate previous findings that showed miR-155 upregulation at 3- and 12-months of the 3xTg-AD mice (Guedes et al., 2014). Interestingly, since SOCS1 is a target of miR-155, increased miR-155 may justify the reduced levels of SOCS1 found in cortex samples of the 3xTg-AD as described above.

The miR-124 is a brain-enriched miRNA, whereas it is particularly expressed in neurons (Jovicic et al., 2013). In microglia, miR-124 targets CEBP-α, a known M1 phenotype marker as described before (Ponomarev et al., 2011a). Although we detected an upregulation of miR-124 in the cortex of 3xTg-AD animals (~1.8-fold, $p < 0.05$), this miR was downregulated at 9-months in both cortical and hippocampal regions (~30% and ~15%, respectively, $p < 0.05$, **Figure 6B**). These results are in accordance with the downregulation of its target CEBP-α seen at 3-months, followed by a tendency for upregulation at 9-months as demonstrated above.

The miR-146a, also considered a brain-enriched miRNA, is thought to be more strongly expressed in microglia than in neurons (Jovicic et al., 2013). Although upregulated in murine microglial cell line LPS (Cunha et al., 2016), miR-146a negatively regulates NF-κB (Taganov et al., 2006) attenuating both the immune and inflammatory responses. Our results show an upregulation of miR-146a in 3-month 3xTg-AD animals at cortex and hippocampus (~1.8- and 1.4-fold, respectively, $p < 0.05$) followed by a downregulation at 9-months (~35% and ~25% for cortex and hippocampus, respectively, $p < 0.05$, **Figure 6C**), which may explain the increase of inflammatory molecules at this later stage. Our results do not corroborate a previous report showing that mir-146a is only upregulated at 10-month 3xTg-AD and 3-months 5xFAD, but not in earlier stages (Li et al., 2011b).

Although all the inflamma-miRs evaluated were increased at the early stage of the 3xTg-AD mice, only miR-155 kept this upregulation for the late stage, suggesting that it may be a continuous marker for AD manifestation. Moreover, since miR-155 is considered a pro-inflammatory molecule that was detected prior to the expression of other inflammatory markers we may hypothesize its potential use as an early biomarker for the emergence of symptomatic AD.

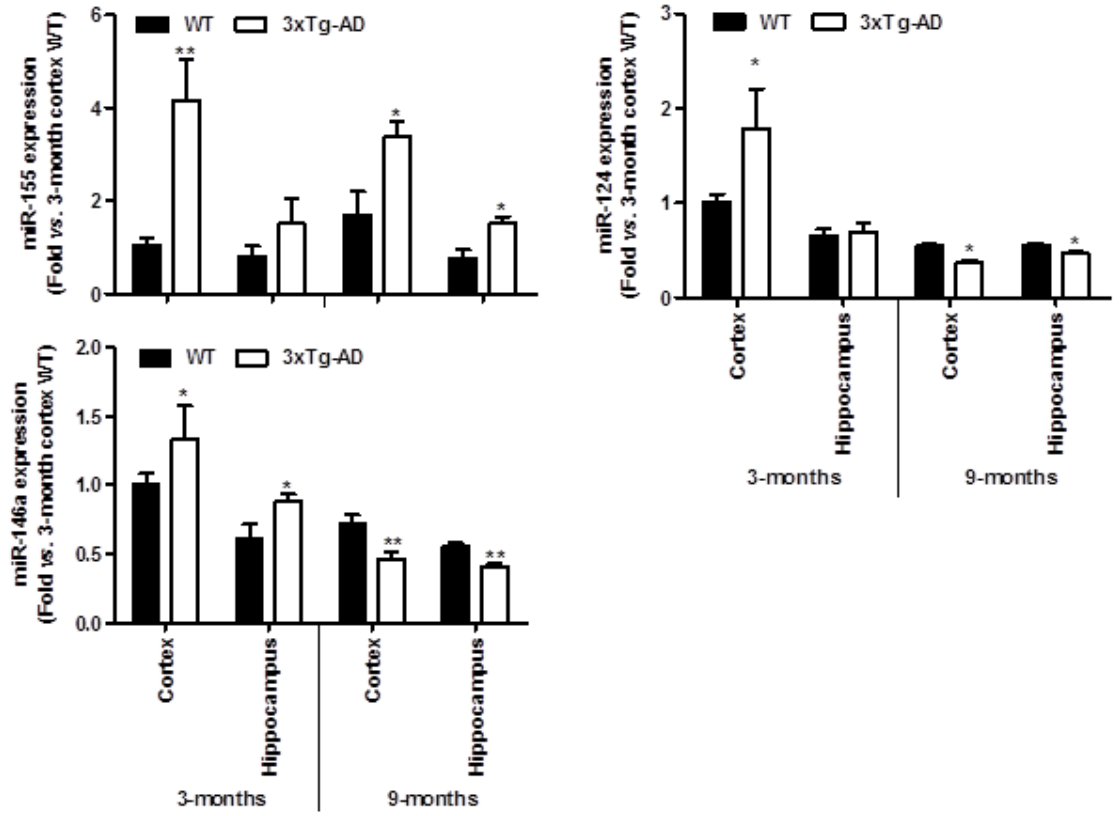

Figure 4. 6 - While all inflamma-miRs are elevated at the early-AD stage of the 3xTg-AD mice, only miR-155 is kept elevated also at 9-months. Brain samples from wild-type (WT) and 3xTg-AD animals were collected at 3- and 9-months and analyzed for **(A)** miR-155, **(B)** miR-124 and **(C)** miR-146a expression by qRT-PCR. Results are expressed as mean ± SEM, representative of n = 4 animals per experimental group and expressed as fold change *vs.* 3-month cortex WT mice. *t*-test $^{*}p < 0.05$, $^{**}p < 0.01$ *vs.* respective WT.

3.6. MiR-155 mainly downregulates protective-associated microglial genes at early-AD stage in the 3xTg-AD mice and even more at 9-months

As miR-155 was found to be a potential biomarker in the early stage of the disease, we decided to explore the expression profile of known target genes regulated by this miR. For that we assessed a pool of samples from WT and 3xTg-AD mice at 3- and 9-months using a miR-155 Targets PCR Array. A preliminary analysis of the results showed that although we used samples from transgenic mice that had increased expression of miR-155 when compared with aged-matched WT, that would supposedly result in downregulation of the respective targets, we still found a few genes that were upregulated. As shown in **Figure 4.7**, a heat map analysis of the expression of miR-155 targets first cluster the WT animals at both animals, then the WT animals with 3-month 3xTg-AD and only after with the 9-month 3xTg-AD. These results clearly show that WT animals have similar expression patterns that are more related with the 3-month

transgenic animal and that 9-month 3xTgAD have the more distinct profile from WT animals.

A more detailed scatter plot analysis of the expression of miR-155 targets in 3-months 3xTg-AD *vs.* WT (**Figure 4.8**) show that transgenic animals have 12 upregulated genes in parallel with 26 downregulated genes, which are listed in **Table 4.2**. Concerning the expression of miR-155 targets in 9-months 3xTg-AD *vs.* WT (**Figure 4.9**) the scatter plot analysis identify that transgenic animals have only 3 upregulated genes and a higher number of 35 downregulated ones, which are listed in **Table 4.2**.

Among the different genes, Septin 11 gene appeared upregulated in both 3- and 9-month 3xTg-AD animals. This gene encodes for a protein member of a conserved family of cytoskeletal GTPases that is involved in vesicle trafficking and may play a role in synaptic connectivity (Roseler et al., 2011). Curiously, it was already reported the presence of transcript variants of Septin 8, another member from the same family, in AD patient samples that favoured the β-amyloidogenic processing of APP by promoting endosome accumulation of BACE1 (Kurkinen et al., 2016). Also MafB gene that is upregulated in 3-months 3xTg-AD animals plays a role in microglia function. MafB is a transcription factor involved in microglia differentiation (Koshida et al., 2015), and maintenance of homeostasis in adulthood, since it microglia-specific knockout lead to increased expression of inflammation pathways (Matcovitch-Natan et al., 2016). So, MafB upregulation may be associated with the observed downregulation of the inflammatory markers in the 3-month transgenic mice as described.

Exploring the downregulated genes we can find the downregulation of the SOCS1 gene in the 9-month 3xTg-AD mice corroborating our results described above. Moreover, the downregulation of CEBP-β gene, that mediates the transcription of anti-inflammatory agents (He et al., 2009;Worm et al., 2009), may justify the observed reduction of M2 markers in the 3-months 3xTg-AD mice. Interestingly, the Runx2 gene, which is involved in microglia phagocytosis and is activated downstream of CEBP-β cascade (Nakazato et al., 2015), was also downregulated in the 3xTg-AD animals, suggesting once more a reduced ability of microglia response in these animals. In addition, the FOXO3 gene was downregulated in both 3- and 9-month 3xTg-AD. However, this survival gene was shown to have contradictory functions along AD progression. While initially activated Foxo3 promotes the MnSOD expression and oxidative stress resistance, continuous activation leads to activation of pro-apoptotic genes Bim and FasL leading to cell death, and also to neurotoxic Aβ processing (Shi et al., 2016).

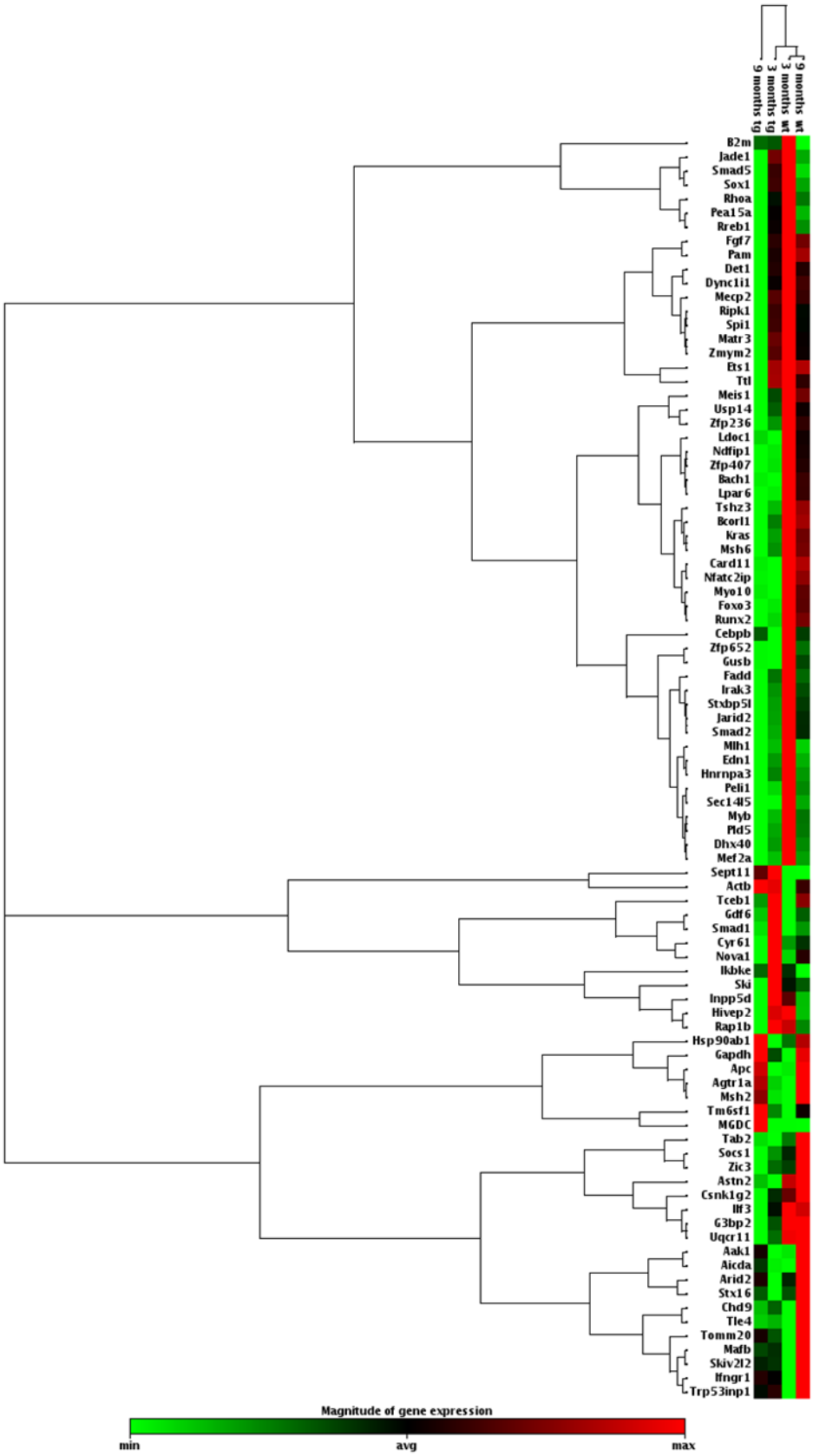

Figure 4. 7 - Heat map of miR-155 Targets PCR Array results for a pool of samples from 3xTg-AD mice (tg) and aged-matched wild-type (WT) mice. RNA from cortical brain samples from WT and 3xTg-AD animals were isolated at 3- and 9-months and analyzed for miR-155 targets gene expression by qRT-PCR.

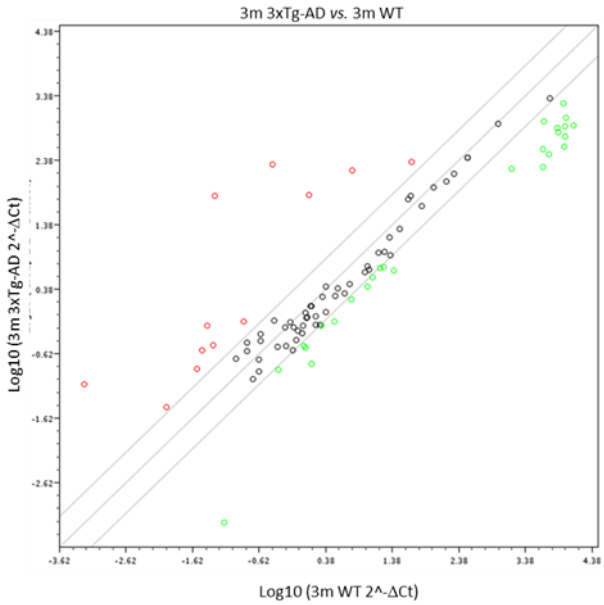

Figure 4. 8 - Scatter plot representation of the expression of miR-155 targets in 3-months 3xTg-AD *vs.* wild-type (WT) mice. RNA from cortical brain samples from WT and 3xTg-AD animals were isolated at 3- and 9-months and analyzed for miR-155 targets gene expression by qRT-PCR.

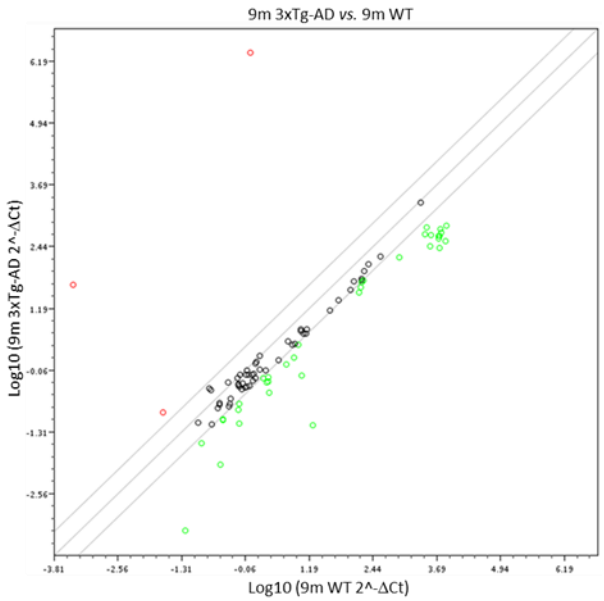

Figure 4. 9 - Scatter plot representation of the expression of miR-155 targets in 9-months 3xTgAD *vs.* wild-type (WT) mice. RNA from cortical brain samples from WT and 3xTg-AD animals were isolated at 3- and 9-months and analyzed for miR-155 targets gene expression by qRT-PCR.

Table 4. 3 - Mouse miR-155 target genes expression at 3-months in the 3xTg-AD *vs.* wild-type (WT) mice

Genes upregulated		Genes downregulated	
Gene	**Fold regulation**	**Gene**	**Fold regulation**
Septin 11	**1314.551**	*Aak1*	-122.87
Tceb1	538.838	*Zfp652*	-23.544
Tm6sf1	143.186	*Sec14l5*	-23.476
Mafb	**51.209**	*Ldoc1*	-17.99
Gdf6	28.071	*Nfatc2ip*	-17.159
Ifngr1	16.511	*Ndfip1*	-15.305
Gapdh	7.956	*Card11*	-12.27
Skiv2l2	6.776	*Lpar6*	-11.942
Smad1	5.384	***Foxo3***	**-11.472**
Cyr61	5.016	***Runx2***	**-9.585**
Nova1	4.829	*Zfp407*	-9.067
Aicda	3.679	*Gusb*	-8.814
		Tab2	-8.525
		Kras	-5.327
		Stxbp5l	-4.763
		Cebpb	**-4.676**
		Mlh1	-4.22
		Peli1	-3.995
		Bach1	-3.84
		Pld5	-3.5
		Hnmpa3	-3.46
		Irak3	-3.412
		Myo10	-3.401
		Zfp236	-3.261
		Mef2a	-3.171
		Fadd	-3.073

Table 4. 4 – Mouse miR-155 target genes expression at 9-months in the 3xTg-AD *vs.* wild-type (WT) mice

Genes overexpressed		Genes underexpressed			
Gene	**Fold regulation**	**Gene**	**Fold regulation**	**Gene**	**Fold regulation**
MGDC	2160132.239	*Kras*	-268.814	*Hnmpa3*	-3.986
Septin11	**131567.175**	*Cyr61*	-113.564	*Tceb1*	-3.966
Ikbke	5.699	*Mecp2*	-26.774	*Irak3*	-3.953
		Runx2	**-21.285**	*Ski*	-3.715
		Ndfip1	-20.299	*Gusb*	-3.605
		Fgf7	-16.183	*Pea15a*	-3.539
		Foxo3	**-13.309**	*Ets1*	-3.248
		Stxbp5l	-12.971	*Bcorl1*	-3.247
		G3bp2	-12.318	*Zic3*	-3.118
		Lpar6	-11.39	*Tle4*	-3.088
		Tab2	-11.381		
		Nfatc2ip	-10.592		
		Zfp407	-9.474		
		Det1	-9.026		
		Rreb1	-8.283		
		Card11	-7.964		
		Zfp652	-5.937		
		Sec14l5	-5.397		
		Zfp236	-5.144		
		Uqcr11	-4.848		
		Fadd	-4.839		
		Dync1i1	-4.614		
		Ldoc1	-4.576		
		Nova1	-4.503		
		Socs1	**-4.497**		

4. Discussion

Experiments in this study were established to assess the temporal profile of microglial reactivity employing the 3xTg-AD mice model of AD, with particular focus on cortex and hippocampus, two of the brain areas most affected by AD (Braak et al., 1993). Here, we observed that microglia refrains their reactivity at very early AD stages (3-months), indicated by reduced expression of inflammatory cytokines, M1 and M2 phenotype markers, while neuroinflammation is increased for later AD stage (9-months). Interestingly, all inflamma-miRs were elevated at the early stage, including the typical M1-related miR-155, which kept upregulated also in the later AD stage, suggesting that it may be an early indicator of the subsequent inflammation and a biomarker of AD.

AD is a progressive and irreversible neurodegenerative disorder that gradually reduces memory and thinking skills, leading to inability to perform simple daily tasks (Rubio-Perez and Morillas-Ruiz, 2012). Alzheimer's brain is characterized by elevated levels of some microglial-derived cytokines suggestive of a chronic inflammatory milieu in the AD brain, which may be neurotoxic and contribute to disease progression (Lopategui Cabezas et al., 2014). AD is complex, and at this moment there is no drug or intervention that successfully cures it. Thus, it is extremely important to understand the causes of Alzheimer's and make an easy diagnosis as early as possible. For these findings, animal models are important tools to identify the molecular markers that trigger the onset of AD-related cognitive decline, as well as to assay potential pharmacological interventions. So, in our work we used the 3xTg-AD mice model of AD which progressively develops both Aβ plaques and neurofibrillary tangles with a temporal and spatial distribution that closely mimics that observed in the human AD brain (Oddo et al., 2003a; Oddo et al., 2003b). The sequence of neuropathological development in this model suggests that Aβ is the initiating trigger of cognitive decline (Oddo et al., 2003a). Therefore, the 3xTg-AD mice model of AD provides a promising tool for understanding the neuropathology of AD, since it express AD pathology in an age-dependent manner, beyond the associated behavioral changes (Sterniczuk et al., 2010) that occur with the disease progress in human patients (Vandenberghe and Tournoy, 2005). Intraneuronal Aβ accumulation is seen in the cortex around 3-months, spreading to the hippocampus and amygdala by 6-month, also when diffuse amyloid plaques are detected in the cerebral cortex (Oddo et al., 2003b; Billings et al., 2005). However, senile plaques widespread are only detected at 12-months (Oddo et al., 2003a; Rodriguez et al., 2010), coupled with the emergence of conformational changes in tau (Oddo et al., 2003b). Our detection of APP/Aβ protein, confirms the expression of high levels of APP right at 3-months in the 3xTg-AD animal, which is maintained along 6- and 9-months.

An increased density of GFAP immunoreactive astrocytes and Iba1 immunoreactive microglia, as well as upregulation of inflammatory markers were shown in 3xTg-AD mice compared with WT at 6/7-months (Janelsins et al., 2008; Caruso et al., 2013), suggesting that it may be a response to Aβ accumulation. Curiously, we found a marked downregulation of the microglia activation marker CD11b at 3-months in cortex and hippocampus of the 3xTg-AD as compared with age-matched WT. Our findings corroborate the previous report by Janelsins and colleagues, showing a decrease F4/80-positive microglia in the hippocampus of 3- and 6-month 3xTg-AD (Janelsins et al., 2005), and that of Rodriguez and colleagues showing an increased density of both resting/ramified and activated microglia in the hippocampus of 9-month 3xTg-AD, prior

to senile plaque spread (Rodriguez et al., 2010). This increased density of ramified microglia may be a preparation phase to refrain the damage and to become prepared to fight the subsequent increased load of extracellular Aβ that is to come. Moreover, ours results are also consistent with data that shown activated microglia almost exclusively surrounding the senile plaques (Rodriguez et al., 2010), which are not detected in the very early stage of animal life that we analyzed (Oddo et al., 2003a; Rodriguez et al., 2010).

It has been described that microglia activation promotes the release of potentially cytotoxic molecules, which contributes to the onset and/or progression of AD (Lopategui Cabezas et al., 2014). Several cytokines, including IL-1β, IL-18 and TNF-α were shown to be overexpressed in AD brain (Ojala et al., 2009; Rubio-Perez and Morillas-Ruiz, 2012). In the present study, and in accordance with the reduced expression of CD11b previously detected, we observed a marked reduction of TNF-α, IL-6, IL-1β and IL-18 essentially in the cortex, and of HMGB1 in the hippocampus, corroborating the reduced activation of microglia in these earlier development of the 3xTg-AD animals. In accordance, other studies using the same 3xTg-AD model also showed a reduction of hippocampal expression of IL-6, TNF-α and IL-1β at 3-months (Janelsins et al., 2005;Guedes et al., 2014), while in the APP/PS1δE9 TNF-α and IL-1β production only occurred after 10-months (Martin et al., 2017), and in the 5xFAD the inflammatory genes were only upregulated after 4-6 months, namely in the hippocampus (Landel et al., 2014). Therefore it seems that an inflammatory response in the different AD animal models is only activated following Aβ accumulation. Curiously, at 9-months 3xTg-AD we observed a higher upregulation of TNF-α, IL-1β and HMGB1 at the hippocampus, suggesting a greater involvement of these cytokines for disease progression. TNF-α and IL-1β have been described for long as mediators of AD pathogenesis (Wang et al., 2015). HMGB1 was reported to accumulate extracellularly on Aβ plaques, being the protein level increased in AD brains (Takata et al., 2003). More recently, HMGB1 was implicated in the pathogenesis of AD by causing a microglial Aβ phagocytosis dysfunction (Takata et al., 2012) and neurite degeneration (Fujita et al., 2016).

Interestingly, a recent study using frontal cortex samples of early AD patients showed 2 distinct populations, one with a typical M1/pro-inflammatoy phenotype with very high expression levels of IL-1β, TNF-α and IL-12A, and other with a M2/resolution of damage polarization with upregulation of IL-1ra, Arginase 1 and FIZZ expression (Sudduth et al., 2013). The authors further associated these phenotypes with the presence of different Aβ species, while M1 phenotype was seen in patients with

increased soluble Aβ, the M2 phenotype was more evident in patients with increased insoluble Aβ (Sudduth et al., 2013), suggesting that oligomeric Aβ acts as a more pro-inflammatory challenge, while senile plaques favor a resolution of damage polarization. Our results clearly show that all M1 and M2 specific markers, except for iNOS, were downregulated in the 3-months 3xTg-AD essentially in the cortex, corroborating once again the refrained microglia reactivity. On the other hand, there was an increase of iNOS, Arginase 1 and SOCS1 in the 9-month 3xTg-AD animals, suggesting a later activation. iNOS, the typical M1 marker, is involved in innate immune response and uses arginine to produce nitric oxide, which has cytotoxic effects (Colton, 2009). On the other hand, the M2 marker Arginase 1 competes with iNOS for the arginine to produce L-ornitine, required for the formation of collagen (Colton, 2009). The activity and protein expression of both iNOS and Arginase 1 were found to be altered with age and in AD (Liu et al., 2014), which justify the later increase observed in our animals.

MiRs are known to be involved in almost all biological functions, to further regulate intracellular processes by targeting multiple mRNA molecules simultaneously, and to control immune cell phenotypes (Guedes et al., 2014). Several studies have reported dysregulation of some miRNAs related with innate immunity and neuroinflammation in AD patient brains (Lukiw et al., 2008; Li et al., 2011a), and with modulation of microglia responses (Su et al., 2015). Recently, miR-155 expression has been described to be altered in AD brain tissue (Culpan et al., 2011) and to enhance neuroinflammation in AD course in a 3xTg-AD animal model of AD (Guedes et al., 2014). The increased level of miR-155 expression that we observed in the 3xTg-AD animal model at early and late stages of the disease is in line with this study. This early miR-155 upregulation in 3xTg-AD animals, in contrast to downregulation of inflammatory biomarkers and microglia activation markers, may be an initial indicator of changes in microglia immune response, and probably contribute to the later production of inflammatory mediators. In addition, the reduction of SOCS1 expression that we observed, in parallel with increased miR-155 expression, agrees with the fact that SOCS1 is a target of miR-155 (Cardoso et al., 2012; Liu and Abraham, 2013). Recent evidence showed that miR-155 was significantly upregulated by HMGB1 mediated by the TLR2/MyD88 pathway (Wen et al., 2013). The significant increase of HMGB1 expression, in both cortical and hippocampal tissues at 9-months may be involved in continuous miR-155 upregulation along disease progression and its potential use as an AD biomarker. MiRs affect neuroimmune functions, by playing important regulatory roles. It was shown that, over an infection and inflammatory process, while miR-155 usually acts to promote microglia-mediated pro-inflammatory responses, miR-146a acts as a negative feedback of inflammation by

inhibiting NF-κB transcriptional activity and the expression of pro-inflammatory cytokines (Su et al., 2015), by acting in TLR signaling (Taganov et al., 2006). The decrease observed in miR-146a expression, accompanied by upregulation of miR-155 during AD progression in 3xTg-AD animals, may be due to negative feedback role that miR-155 has over miR-146a. Also miR-124 was related to downregulation of activated microglia (Ponomarev et al., 2013), contributing to the surveillance/quiescent state (M0) of microglia (Caldeira et al., 2014) by acting on its target CEBP-α (Ponomarev et al., 2011a). Indeed, we found an upregulation of miR-124 at 3-months in 3xTg-AD mice, which is in accordance with the reduced CEBP-α expression observed. In addition, Ponomarev and colleagues related that upregulation of miR-124 promote microglia M2-phenotype by downregulation of M1-associated markers, like TNF-α, iNOS and MHC class II (Ponomarev et al., 2011a), corroborating our findings. However, the increase of M2-related markers that they have found, such as TGF-β and Arginase 1, was not observed in our work.

As already mentioned, miR-155 can regulate many cellular functions by modulating multiple targets. Looking at the expression of some miR-155 validated and predicted targets, it was interesting to see that, besides all the individual variation that we had in our previous results, control animals clustered together representing an increased similarity in their expression profile, followed by 3-month 3xTg-AD and then the 9-month transgenic animal. These preliminary data, that need to be verified, suggest that changes between the 3xTg-AD and aged-matched WT animals increase with age. We further observed that besides we looked at samples where the miR-155 was elevated when compared to WT, we still have upregulated targets in the transgenic animals but in lower amount than the downregulated ones. Curiously, a first analysis showed an altered expression of some genes that have been implicated in microglia reactivity and AD. Indeed, genes from Septin family, are involved in vesicle trafficking (Roseler et al., 2011), and favor the β-amyloidogenic processing of APP through BACE1 accumulation (Kurkinen et al., 2016), while MafB gene is involved in microglia inflammatory control (Matcovitch-Natan et al., 2016). On the other hand, CEBP-β gene was confirmed to be a direct target of mir-155 in this AD model, as previously described (Guedes et al., 2014). Furthermore, Runx2 gene is involved in microglia phagocytosis (Nakazato et al., 2015), while FOXO3 gene is involved in an earlier oxidative stress resistance but later apoptosis induction and neurotoxic Aβ processing (Shi et al., 2016). These findings open new avenues for further target evaluation concerning distinct microglia reactivity along AD onset and progression.

Overall, our results suggest that microglia at 3-months of 3xTg-AD animals has a more irresponsive phenotype, probably being in a more surveillance/quiescent stage. In fact, a recent study on transcriptional changes in AD shows a global downregulation of the expression of the genes that encode proteins that are metastable to aggregation (Ciryam et al., 2016). This downregulation may help to attenuate anomalous protein aggregation when protein homeostasis becomes compromised in AD (Ciryam et al., 2016). Meanwhile, upregulation of both miR-155, TNF-α, IL-1β and HMGB1 found at 9-months in 3xTg-AD mice together with downregulation of miR-124 and miR-146a, indicates a more pro-inflammatory microglial response at this stage of disease. These age-dependent neuroinflammatory changes may be critical in cognitive decline in AD (Olah et al., 2011), which may explain the fact that treatments with NSAIDs only act to prevent disease progression when initiated at an earlier stage of inflammation establishment (Varvel et al., 2009; Krause and Muller, 2010).

5. Conclusions

Overall, our results suggest an immunodepressed status of microglia, in the 3xTg-AD animal model, at initial phase of disease (3-months), as if in preparation for the ensuing activation process to combat the subsequent extracellular Aβ accumulation characteristic of the later stages of AD. This deregulated microglia response at the early stages of disease may result to an exacerbated disease progression. Interestingly, miR-155 revealed to be an early biomarker of inflammation in AD. Thereby, microglia cells are phenotypically and functionally distinct during disease course reinforcing the need of distinct therapeutic strategies depending on the disease stage (**Figure 4.10**).

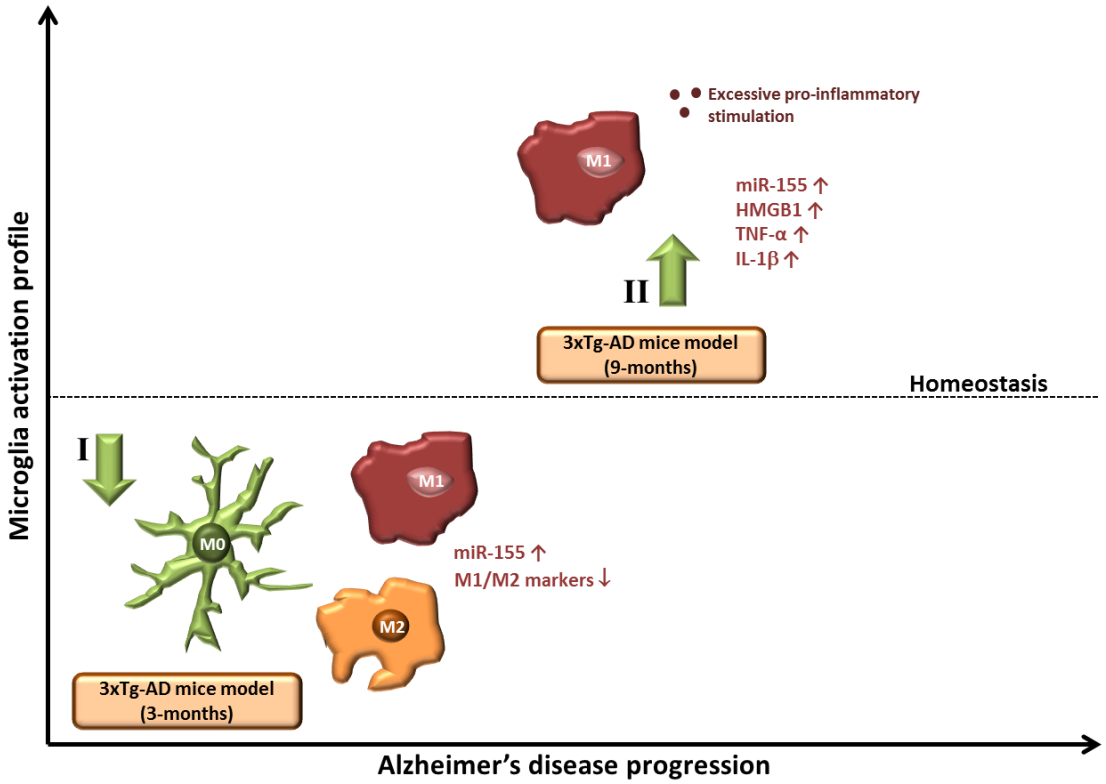

Figure 4. 10 - Microglia activation profile during Alzheimer's disease (AD) progression in the 3xTg-AD mice model. Microglial cells showed an attenuated response in disease onset (3-months) in 3xTg-AD mice, presenting a resting/quiescent state (M0) and without prevalence of a pro-inflammatory M1-phenotype (classical activation) or anti-inflammatory M2-phenotype (alternative activation). The upregulation of pro-inflammatory microRNA-155 (miR-155) at this stage, revealed to be an early biomarker of inflammation in AD. At 9-months it was observed increased microglia reactivity (M1-phenotype) and the presence of an inflammatory milieu with upregulation of inflammatory-mediators and miR-155 (II). This distinct microglia reactivity along AD course highlights the need for different therapeutic approaches depending on the stage of disease.

6. References

Alexandrov, P.N., Dua, P., Hill, J.M., Bhattacharjee, S., Zhao, Y., and Lukiw, W.J. (2012). microRNA (miRNA) speciation in Alzheimer's disease (AD) cerebrospinal fluid (CSF) and extracellular fluid (ECF). *Int J Biochem Mol Biol* 3**,** 365-373.

Bachstetter, A.D., Van Eldik, L.J., Schmitt, F.A., Neltner, J.H., Ighodaro, E.T., Webster, S.J., Patel, E., Abner, E.L., Kryscio, R.J., and Nelson, P.T. (2015). Disease-related microglia heterogeneity in the hippocampus of Alzheimer's disease, dementia with Lewy bodies, and hippocampal sclerosis of aging. *Acta Neuropathol Commun* 3**,** 32.

Barateiro, A., Miron, V.E., Santos, S.D., Relvas, J.B., Fernandes, A., Ffrench-Constant, C., and Brites, D. (2013). Unconjugated bilirubin restricts oligodendrocyte differentiation and axonal myelination. *Mol Neurobiol* 47**,** 632-644.

Barateiro, A., Vaz, A.R., Silva, S.L., Fernandes, A., and Brites, D. (2012). ER stress, mitochondrial dysfunction and calpain/JNK activation are involved in oligodendrocyte precursor cell death by unconjugated bilirubin. *Neuromolecular Med* 14**,** 285-302.

Billings, L.M., Oddo, S., Green, K.N., Mcgaugh, J.L., and Laferla, F.M. (2005). Intraneuronal Abeta causes the onset of early Alzheimer's disease-related cognitive deficits in transgenic mice. *Neuron* 45**,** 675-688.

Braak, H., Braak, E., and Bohl, J. (1993). Staging of Alzheimer-related cortical destruction. *Eur Neurol* 33**,** 403-408.

Cai, H., Liang, Q., and Ge, G. (2016). Gypenoside Attenuates beta Amyloid-Induced Inflammation in N9 Microglial Cells via SOCS1 Signaling. *Neural Plast* 2016**,** 6362707.

Caldeira, C., Cunha, C., Vaz, A.R., Falcão, A.S., Barateiro, A., Seixas, E., Fernandes, A., and Brites, D. (2017). Key aging-associated alterations in primary microglia response to beta-amyloid stimulation. *Submitted*.

Caldeira, C., Oliveira, A.F., Cunha, C., Vaz, A.R., Falcão, A.S., Fernandes, A., and Brites, D. (2014). Microglia change from a reactive to an age-like phenotype with the time in culture. *Front Cell Neurosci* 8**,** 152.

Cardoso, A.L., Guedes, J.R., Pereira De Almeida, L., and Pedroso De Lima, M.C. (2012). miR-155 modulates microglia-mediated immune response by down-regulating SOCS-1 and promoting cytokine and nitric oxide production. *Immunology* 135**,** 73-88.

Caruso, D., Barron, A.M., Brown, M.A., Abbiati, F., Carrero, P., Pike, C.J., Garcia-Segura, L.M., and Melcangi, R.C. (2013). Age-related changes in neuroactive steroid levels in 3xTg-AD mice. *Neurobiol Aging* 34**,** 1080-1089.

Ciryam, P., Kundra, R., Freer, R., Morimoto, R.I., Dobson, C.M., and Vendruscolo, M. (2016). A transcriptional signature of Alzheimer's disease is associated with a metastable subproteome at risk for aggregation. *Proc Natl Acad Sci U S A* 113**,** 4753-4758.

Colton, C.A. (2009). Heterogeneity of microglial activation in the innate immune response in the brain. *J Neuroimmune Pharmacol* 4**,** 399-418.

Colton, C.A., Mott, R.T., Sharpe, H., Xu, Q., Van Nostrand, W.E., and Vitek, M.P. (2006). Expression profiles for macrophage alternative activation genes in AD and in mouse models of AD. *J Neuroinflammation* 3**,** 27.

Culpan, D., Kehoe, P.G., and Love, S. (2011). Tumour necrosis factor-alpha (TNF-alpha) and miRNA expression in frontal and temporal neocortex in Alzheimer's disease and the effect of TNF-alpha on miRNA expression in vitro. *Int J Mol Epidemiol Genet* 2**,** 156-162.

Cummings, J.L., Morstorf, T., and Zhong, K. (2014). Alzheimer's disease drug-development pipeline: few candidates, frequent failures. *Alzheimers Res Ther* 6**,** 37.

Cunha, C., Gomes, C., Vaz, A.R., and Brites, D. (2016). Exploring New Inflammatory Biomarkers and Pathways during LPS-Induced M1 Polarization. *Mediators Inflamm* 2016**,** 6986175.

Drummond, E., and Wisniewski, T. (2017). Alzheimer's disease: experimental models and reality. *Acta Neuropathol* 133**,** 155-175.

Duyckaerts, C., Potier, M.C., and Delatour, B. (2008). Alzheimer disease models and human neuropathology: similarities and differences. *Acta Neuropathol* 115**,** 5-38.

Eimer, W.A., and Vassar, R. (2013). Neuron loss in the 5XFAD mouse model of Alzheimer's disease correlates with intraneuronal Abeta42 accumulation and Caspase-3 activation. *Mol Neurodegener* 8**,** 2.

Fujita, K., Motoki, K., Tagawa, K., Chen, X., Hama, H., Nakajima, K., Homma, H., Tamura, T., Watanabe, H., Katsuno, M., et al. (2016). HMGB1, a pathogenic molecule that induces neurite degeneration via TLR4-MARCKS, is a potential therapeutic target for Alzheimer's disease. *Sci Rep* 6**,** 31895.

Guedes, J.R., Custodia, C.M., Silva, R.J., De Almeida, L.P., Pedroso De Lima, M.C., and Cardoso, A.L. (2014). Early miR-155 upregulation contributes to neuroinflammation in Alzheimer's disease triple transgenic mouse model. *Hum Mol Genet* 23**,** 6286-6301.

He, M., Xu, Z.Q., Ding, T., Kuang, D.M., and Zheng, L.M. (2009). MicroRNA-155 Regulates Inflammatory Cytokine Production in Tumor-associated Macrophages via Targeting C/EBP beta. *Cellular & Molecular Immunology* 6**,** 343-352.

Howlett, D.R., and Richardson, J.C. (2009). The pathology of APP transgenic mice: a model of Alzheimer's disease or simply overexpression of APP? *Histol Histopathol* 24**,** 83-100.

Iwahara, N., Hisahara, S., Kawamata, J., Matsumura, A., Yokokawa, K., Saito, T., Fujikura, M., Manabe, T., Suzuki, H., Matsushita, T., et al. (2017). Role of Suppressor of Cytokine Signaling 3 (SOCS3) in Altering Activated Microglia Phenotype in APPswe/PS1dE9 Mice. *J Alzheimers Dis* 55**,** 1235-1247.

Janelsins, M.C., Mastrangelo, M.A., Oddo, S., Laferla, F.M., Federoff, H.J., and Bowers, W.J. (2005). Early correlation of microglial activation with enhanced tumor necrosis factor-alpha and monocyte chemoattractant protein-1 expression specifically within the entorhinal cortex of triple transgenic Alzheimer's disease mice. *Journal of neuroinflammation* 2**,** 23.

Janelsins, M.C., Mastrangelo, M.A., Park, K.M., Sudol, K.L., Narrow, W.C., Oddo, S., Laferla, F.M., Callahan, L.M., Federoff, H.J., and Bowers, W.J. (2008). Chronic neuron-specific tumor necrosis factor-alpha expression enhances the local inflammatory environment ultimately leading to neuronal death in 3xTg-AD mice. *Am J Pathol* 173**,** 1768-1782.

Jankowsky, J.L., Fadale, D.J., Anderson, J., Xu, G.M., Gonzales, V., Jenkins, N.A., Copeland, N.G., Lee, M.K., Younkin, L.H., Wagner, S.L., et al. (2004). Mutant presenilins specifically elevate the levels of the 42 residue beta-amyloid peptide in vivo: evidence for augmentation of a 42-specific gamma secretase. *Hum Mol Genet* 13**,** 159-170.

Jovicic, A., Roshan, R., Moisoi, N., Pradervand, S., Moser, R., Pillai, B., and Luthi-Carter, R. (2013). Comprehensive Expression Analyses of Neural Cell-Type-Specific miRNAs Identify New Determinants of the Specification and Maintenance of Neuronal Phenotypes. *Journal of Neuroscience* 33**,** 5127-5137.

Koshida, R., Oishi, H., Hamada, M., and Takahashi, S. (2015). MafB antagonizes phenotypic alteration induced by GM-CSF in microglia. *Biochem Biophys Res Commun* 463**,** 109-115.

Krause, D.L., and Muller, N. (2010). Neuroinflammation, microglia and implications for anti-inflammatory treatment in Alzheimer's disease. *Int J Alzheimers Dis* 2010.

Kurkinen, K.M., Marttinen, M., Turner, L., Natunen, T., Makinen, P., Haapalinna, F., Sarajarvi, T., Gabbouj, S., Kurki, M., Paananen, J., et al. (2016). SEPT8

modulates beta-amyloidogenic processing of APP by affecting the sorting and accumulation of BACE1. *J Cell Sci* 129**,** 2224-2238.

Landel, V., Baranger, K., Virard, I., Loriod, B., Khrestchatisky, M., Rivera, S., Benech, P., and Feron, F. (2014). Temporal gene profiling of the 5XFAD transgenic mouse model highlights the importance of microglial activation in Alzheimer's disease. *Mol Neurodegener* 9**,** 33.

Li, Y.Y., Cui, J.G., Dua, P., Pogue, A.I., Bhattacharjee, S., and Lukiw, W.J. (2011a). Differential expression of miRNA-146a-regulated inflammatory genes in human primary neural, astroglial and microglial cells. *Neurosci Lett* 499**,** 109-113.

Li, Y.Y., Cui, J.G., Hill, J.M., Bhattacharjee, S., Zhao, Y., and Lukiw, W.J. (2011b). Increased expression of miRNA-146a in Alzheimer's disease transgenic mouse models. *Neurosci Lett* 487**,** 94-98.

Liu, G., and Abraham, E. (2013). MicroRNAs in immune response and macrophage polarization. *Arterioscler Thromb Vasc Biol* 33**,** 170-177.

Liu, P., Fleete, M.S., Jing, Y., Collie, N.D., Curtis, M.A., Waldvogel, H.J., Faull, R.L., Abraham, W.C., and Zhang, H. (2014). Altered arginine metabolism in Alzheimer's disease brains. *Neurobiol Aging* 35**,** 1992-2003.

Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25**,** 402-408.

Lopategui Cabezas, I., Herrera Batista, A., and Penton Rol, G. (2014). The role of glial cells in Alzheimer disease: potential therapeutic implications. *Neurologia* 29**,** 305-309.

Lukiw, W.J., Zhao, Y., and Cui, J.G. (2008). An NF-kappaB-sensitive micro RNA-146a-mediated inflammatory circuit in Alzheimer disease and in stressed human brain cells. *J Biol Chem* 283**,** 31315-31322.

Martin, E., Boucher, C., Fontaine, B., and Delarasse, C. (2017). Distinct inflammatory phenotypes of microglia and monocyte-derived macrophages in Alzheimer's disease models: effects of aging and amyloid pathology. *Aging Cell* 16**,** 27-38.

Matcovitch-Natan, O., Winter, D.R., Giladi, A., Vargas Aguilar, S., Spinrad, A., Sarrazin, S., Ben-Yehuda, H., David, E., Zelada Gonzalez, F., Perrin, P., et al. (2016). Microglia development follows a stepwise program to regulate brain homeostasis. *Science* 353**,** aad8670.

Mizuno, T. (2012). The biphasic role of microglia in Alzheimer's disease. *Int J Alzheimers Dis* 2012**,** 737846.

Morrissette, D.A., Parachikova, A., Green, K.N., and Laferla, F.M. (2009). Relevance of transgenic mouse models to human Alzheimer disease. *J Biol Chem* 284**,** 6033-6037.

Nakazato, R., Takarada, T., Ikeno, S., Nakamura, S., Kutsukake, T., Hinoi, E., and Yoneda, Y. (2015). Upregulation of Runt-Related Transcription Factor-2 Through CCAAT Enhancer Binding Protein-beta Signaling Pathway in Microglial BV-2 Cells Exposed to ATP. *J Cell Physiol* 230**,** 2510-2521.

Oakley, H., Cole, S.L., Logan, S., Maus, E., Shao, P., Craft, J., Guillozet-Bongaarts, A., Ohno, M., Disterhoft, J., Van Eldik, L., et al. (2006). Intraneuronal beta-amyloid aggregates, neurodegeneration, and neuron loss in transgenic mice with five familial Alzheimer's disease mutations: potential factors in amyloid plaque formation. *J Neurosci* 26**,** 10129-10140.

Oddo, S., Caccamo, A., Kitazawa, M., Tseng, B.P., and Laferla, F.M. (2003a). Amyloid deposition precedes tangle formation in a triple transgenic model of Alzheimer's disease. *Neurobiol Aging* 24**,** 1063-1070.

Oddo, S., Caccamo, A., Shepherd, J.D., Murphy, M.P., Golde, T.E., Kayed, R., Metherate, R., Mattson, M.P., Akbari, Y., and Laferla, F.M. (2003b). Triple-transgenic model of Alzheimer's disease with plaques and tangles: intracellular Abeta and synaptic dysfunction. *Neuron* 39**,** 409-421.

Ojala, J., Alafuzoff, I., Herukka, S.K., Van Groen, T., Tanila, H., and Pirttila, T. (2009). Expression of interleukin-18 is increased in the brains of Alzheimer's disease patients. *Neurobiol Aging* 30**,** 198-209.

Olah, M., Biber, K., Vinet, J., and Boddeke, H.W. (2011). Microglia phenotype diversity. *CNS Neurol Disord Drug Targets* 10**,** 108-118.

Ponomarev, E.D., Veremeyko, T., Barteneva, N., Krichevsky, A.M., and Weiner, H.L. (2011a). MicroRNA-124 promotes microglia quiescence and suppresses EAE by deactivating macrophages via the C/EBP-alpha-PU.1 pathway. *Nat Med* 17**,** 64-70.

Ponomarev, E.D., Veremeyko, T., Barteneva, N., Krichevsky, A.M., and Weiner, H.L. (2011b). MicroRNA-124 promotes microglia quiescence and suppresses EAE by deactivating macrophages via the C/EBP-alpha-PU.1 pathway. *Nature Medicine* 17**,** 64-U234.

Ponomarev, E.D., Veremeyko, T., and Weiner, H.L. (2013). MicroRNAs are universal regulators of differentiation, activation, and polarization of microglia and macrophages in normal and diseased CNS. *Glia* 61**,** 91-103.

Richartz, E., Stransky, E., Batra, A., Simon, P., Lewczuk, P., Buchkremer, G., Bartels, M., and Schott, K. (2005). Decline of immune responsiveness: a pathogenetic factor in Alzheimer's disease? *J Psychiatr Res* 39**,** 535-543.

Rodriguez, J.J., Witton, J., Olabarria, M., Noristani, H.N., and Verkhratsky, A. (2010). Increase in the density of resting microglia precedes neuritic plaque formation and microglial activation in a transgenic model of Alzheimer's disease. *Cell Death Dis* 1**,** e1.

Roseler, S., Sandrock, K., Bartsch, I., Busse, A., Omran, H., Loges, N.T., and Zieger, B. (2011). Lethal phenotype of mice carrying a Sept11 null mutation. *Biol Chem* 392**,** 779-781.

Roszer, T. (2015). Understanding the Mysterious M2 Macrophage through Activation Markers and Effector Mechanisms. *Mediators Inflamm* 2015**,** 816460.

Rubio-Perez, J.M., and Morillas-Ruiz, J.M. (2012). A review: inflammatory process in Alzheimer's disease, role of cytokines. *ScientificWorldJournal* 2012**,** 756357.

Schwab, C., Klegeris, A., and Mcgeer, P.L. (2009). Inflammation in transgenic mouse models of neurodegenerative disorders. *Biochim Biophys Acta* 1802**,** 889-902.

Schwartz, M., Butovsky, O., Bruck, W., and Hanisch, U.K. (2006). Microglial phenotype: is the commitment reversible? *Trends Neurosci* 29**,** 68-74.

Shi, C., Viccaro, K., Lee, H.G., and Shah, K. (2016). Cdk5-Foxo3 axis: initially neuroprotective, eventually neurodegenerative in Alzheimer's disease models. *J Cell Sci* 129**,** 1815-1830.

Simões, A.E., Pereira, D.M., Amaral, J.D., Nunes, A.F., Gomes, S.E., Rodrigues, P.M., Lo, A.C., D'hooge, R., Steer, C.J., Thibodeau, S.N., et al. (2013). Efficient recovery of proteins from multiple source samples after TRIzol((R)) or TRIzol((R))LS RNA extraction and long-term storage. *BMC Genomics* 14**,** 181.

Sterniczuk, R., Dyck, R.H., Laferla, F.M., and Antle, M.C. (2010). Characterization of the 3xTg-AD mouse model of Alzheimer's disease: part 1. Circadian changes. *Brain Res* 1348**,** 139-148.

Su, W., Aloi, M.S., and Garden, G.A. (2015). MicroRNAs mediating CNS inflammation: Small regulators with powerful potential. *Brain Behav Immun*.

Sudduth, T.L., Schmitt, F.A., Nelson, P.T., and Wilcock, D.M. (2013). Neuroinflammatory phenotype in early Alzheimer's disease. *Neurobiol Aging* 34**,** 1051-1059.

Taganov, K.D., Boldin, M.P., Chang, K.J., and Baltimore, D. (2006). NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. *Proc Natl Acad Sci U S A* 103**,** 12481-12486.

Takata, K., Kitamura, Y., Kakimura, J., Shibagaki, K., Tsuchiya, D., Taniguchi, T., Smith, M.A., Perry, G., and Shimohama, S. (2003). Role of high mobility group protein-

1 (HMG1) in amyloid-beta homeostasis. *Biochem Biophys Res Commun* 301**,** 699-703.

Takata, K., Takada, T., Ito, A., Asai, M., Tawa, M., Saito, Y., Ashihara, E., Tomimoto, H., Kitamura, Y., and Shimohama, S. (2012). Microglial Amyloid-beta1-40 Phagocytosis Dysfunction Is Caused by High-Mobility Group Box Protein-1: Implications for the Pathological Progression of Alzheimer's Disease. *Int J Alzheimers Dis* 2012**,** 685739.

Vandenberghe, R., and Tournoy, J. (2005). Cognitive aging and Alzheimer's disease. *Postgrad Med J* 81**,** 343-352.

Varvel, N.H., Bhaskar, K., Kounnas, M.Z., Wagner, S.L., Yang, Y., Lamb, B.T., and Herrup, K. (2009). NSAIDs prevent, but do not reverse, neuronal cell cycle reentry in a mouse model of Alzheimer disease. *J Clin Invest* 119**,** 3692-3702.

Volianskis, A., Kostner, R., Molgaard, M., Hass, S., and Jensen, M.S. (2010). Episodic memory deficits are not related to altered glutamatergic synaptic transmission and plasticity in the CA1 hippocampus of the APPswe/PS1deltaE9-deleted transgenic mice model of ss-amyloidosis. *Neurobiol Aging* 31**,** 1173-1187.

Walker, D.G., and Lue, L.F. (2015). Immune phenotypes of microglia in human neurodegenerative disease: challenges to detecting microglial polarization in human brains. *Alzheimers Res Ther* 7**,** 56.

Wang, W.X., Huang, Q., Hu, Y., Stromberg, A.J., and Nelson, P.T. (2011). Patterns of microRNA expression in normal and early Alzheimer's disease human temporal cortex: white matter versus gray matter. *Acta Neuropathol* 121**,** 193-205.

Wang, W.Y., Tan, M.S., Yu, J.T., and Tan, L. (2015). Role of pro-inflammatory cytokines released from microglia in Alzheimer's disease. *Ann Transl Med* 3**,** 136.

Wen, Z., Xu, L., Chen, X., Xu, W., Yin, Z., Gao, X., and Xiong, S. (2013). Autoantibody induction by DNA-containing immune complexes requires HMGB1 with the TLR2/microRNA-155 pathway. *J Immunol* 190**,** 5411-5422.

Whyte, C.S., Bishop, E.T., Ruckerl, D., Gaspar-Pereira, S., Barker, R.N., Allen, J.E., Rees, A.J., and Wilson, H.M. (2011). Suppressor of cytokine signaling (SOCS)1 is a key determinant of differential macrophage activation and function. *J Leukoc Biol* 90**,** 845-854.

Wilcock, D.M. (2012). A changing perspective on the role of neuroinflammation in Alzheimer's disease. *Int J Alzheimers Dis* 2012**,** 495243.

Worm, J., Stenvang, J., Petri, A., Frederiksen, K.S., Obad, S., Elmen, J., Hedtjarn, M., Straarup, E.M., Hansen, J.B., and Kauppinen, S. (2009). Silencing of microRNA-155 in mice during acute inflammatory response leads to derepression of c/ebp Beta and down-regulation of G-CSF. *Nucleic Acids Research* 37**,** 5784-5792.

Zou, C., Shi, Y., Ohli, J., Schuller, U., Dorostkar, M.M., and Herms, J. (2016). Neuroinflammation impairs adaptive structural plasticity of dendritic spines in a preclinical model of Alzheimer's disease. *Acta Neuropathol* 131**,** 235-246.

Chapter 5

FINAL CONSIDERATIONS

1. Concluding Remarks and Perspectives

The main goal of this thesis was to explore how different microglia phenotypes and aging may influence Alzheimer's disease (AD) pathogenesis and neuroinflammation. We assessed the microglia reactivity of 2 days *in vitro* (DIV) (activated) and 16 DIV (aged) cells upon treatment with 50 nM and 1000 nM Aβ for 24 h, and the inflammatory status of cortical and hippocampal samples from triple transgenic mice-AD (3xTg-AD) at 3, 6 and 9 months-old. Globally, we observed that the recently isolated microglia behave as an activated cell, while that maintained longer time in culture showed an irresponsive phenotype. When treated with Aβ these cells revealed the existence of key-aging associated alterations, with a predominant microglia M1 polarization and loss of phagocytic ability in the 2 DIV cells, together with a decreased reactivity of the 16 DIV microglia towards the stressful stimulus. In addition, we further observed that in early neuropathogenesis, the 3 months-old 3xTg-AD mice show a down-regulated inflammatory status that progress to a dysfunctional inflammatory grade in the 9 months animals. Data suggest the existence of diverse pathological stages along AD progression that may require different disease-modifying therapies.

AD is pathologically characterized by the presence of extracellular accumulation of Aβ peptides into amyloid plaques, intracellular neurofibrillary tangles (NFTs), neurodegeneration, neuroinflammation and cognitive dysfunction (Rezai-Zadeh et al., 2011; Ardestani et al., 2017). Chronic neurodegeneration leads to microglial activation, however the contribution of inflammation to the disease progression remains to be clarified (Cunningham et al., 2009). In fact, the effect of non-steroidal anti-inflammatory drugs (NSAIDs) has only been shown to be beneficial against the development of AD if administered prior to the development of neurodegeneration (Weggen et al., 2001; Etminan et al., 2003), and may be harmful if given at later stages of the disease (Martin et al., 2008). Furthermore, some studies reported that microglia become more senescent/dystrophic (Graeber and Streit, 2010), as well as less reactive to stimuli, with age (Njie et al., 2012; Streit and Xue, 2012), leading to the loss of their neuroprotective capacities which may increase susceptibility and promote neurodegeneration with AD progression.

Over time, several studies have been carried out in order to evaluate and better understand the response and behavior of microglial cells with ageing and AD progression. Several *in vitro* and *in vivo* experimental models, and brain samples from AD patients, have been used, but it has not yet been possible to define which model better mimics what happens in the human brain. *In vitro* models of microglia, cell lines,

or primary microglia obtained from embryos (Gingras et al., 2007) and newborn animals (Floden and Combs, 2007), do not mimic the adult or aged behavior of microglial cells (Sierra et al., 2007; Harry, 2013). The same happens when using microglia obtained from animals of different ages (von Bernhardi, 2010; Njie et al., 2012; Lai et al., 2013), which, in addition to be very time-consuming, also does not correctly reproduce microglia senescence. These facts highlight the need to set up an experimental model that allows the evaluation how a mild activated microglia respond to a further Aβ stimulus, in comparison with an aged more irresponsive cell, a difficult issue if we consider that there are no protocols to isolate degenerative microglia for study (Njie et al., 2012).

This led us to develop an *in vitro* model to age naturally primary microglial cells *in vitro*, in order to characterize the behaviour and changes of cultured microglia over time in culture, as well as to evaluate whether their response to Aβ stimulus is altered by the age of the cells. This model although established the phenotype acquired by the acutely isolated and activated microglia when treated Aβ, allows the comparison with the correspondent aged-like cell. After understanding the effects of ageing and Aβ exposure in *in vitro* microglia, we performed *in vivo* studies to better evaluate microglial response and its influence for the inflammatory status at differently aged animals, using a triple transgenic AD (3xTg-AD) mouse model.

In the first study (**Chapter 2**) we demonstrated that microglia isolated from newborn mice and maintained in *in vitro* for long term culture change from an activated/reactive phenotype (2 DIV) to a more irresponsive one (16 DIV) with ageing-associated changes, as it was observed in *in vitro* ageing of astrocytes and neurons (Falcão et al., 2005, 2006). So, this study was pioneer in ageing primary microglia cells in *in vitro* cultures. Our data show that cultured aged microglia changes their amoeboid morphology to a more ramified shape indicative of a less activated phenotype with age. This is in line with results obtained by Lai and colleagues who observed that microglia isolated from adult and aged animals have a propensity to acquire a more ramified morphology (Lai et al., 2013). Our aged microglia also exhibited cells with a bipolar shape and shorter large processes that are signs of microglia senescence (Streit et al., 2004). These morphological changes are accompanied by diminished basal nuclear factor-kappaB (NF-κB) activation, like a marked downregulation observed in cultured senescent human WI-38 fibroblasts (Helenius et al., 1996). Thus, our cultured aged microglia appear to acquire a dystrophic and irresponsive phenotype as observed in mice with AD-associated disorder (Krabbe et al., 2013). Also the impaired migration capacity of microglia observed with time in culture is in line with studies showing that aged microglia

become less dynamic with slower acute responses and lower rates of process motility (Damani et al., 2011). In addition, our *in vitro* aged microglia show an impaired ability to phagocytose, probably due to the presence of a senescent phenotype, that is in accordance with data obtained with microglia from aged mice that internalized less Aβ than microglia from neonatal or young mice (Njie et al., 2012). This is also related with the low expression of TLR2 and TLR4 that mediate the phagocytosis of infectious pathogens (Napoli and Neumann, 2009). The reduced efflux of glutamate we obtained in aged microglia cultures is in line with age-dependent decline of glutamate release in mice (Minkeviciene et al., 2008). Similarly, the decreased activation of MMP9 we observed in aged microglia was also noticed by others (Bonnema et al., 2007; Paczek et al., 2008). This study was the first to provide the inflamma-miRNA signature for microglial ageing in primary cultures. Microglia showed increased miR-146a expression and SA-β-gal activity, consistent with the existence of senescent cells in aged microglia culture. In fact, miR-146a was implicated in age-related dysfunction of macrophages (Jiang et al., 2012). Additionally, reduced expression of miR-155 and miR-124 that revealed a negative correlation with age (Fichtlscherer et al., 2010; Noren Hooten et al., 2010; Smith-Vikos and Slack, 2012), reinforces that cultured microglial cells at 16 DIV mainly represent an increased population of aged-associated microglia subclass. Moreover, the decreased autophagic capacity we observed in 16 DIV microglia was also shown to occur by ageing (Bergamini, 2006), further suggesting the presence of a dystrophic and irresponsive phenotype characteristic of aged microglia, in our experimental model.

Altogether, this chapter describes the development and characterization of an *in vitro* model to age microglial cells, allowing a better characterization of such microglia subtype, in addition to the 2 DIV microglia, more closely representing a mild activated state.

In **Chapter 3** we evaluated the response of primary microglia cultured in our *in vitro* ageing model when exposed to Aβ stimulation. We did not observe significant age-dependent cell death changes, either in the presence or absence of Aβ stimulus, reinforcing the validity of our model. Thus, the impairment of microglial neuroprotective functions, such as migration, phagocytosis and autophagy observed in the study is a result of *in vitro* ageing. Furthermore, we demonstrated that microglial response to Aβ depends on the activation state of the cell. If microglia are already activated they respond with increased migration, probably due to the release of ATP promoted by both fibrillar and oligomeric $A\beta_{1-42}$ forms used (Kim et al., 2012), as well as expression of most of the

pro-inflammatory biomarkers, with the exception of NLRP3, suggesting a higher neuroinflammatory level of the young microglia *vs* the aged one. However, these cells showed a diminished inflammatory miR-155 and miR-124 expression as a result of Aβ interaction, as well as decreased phagocytosis, indicating a loss of function as observed in other studies (Neumann et al., 2009; Li, 2012; Njie et al., 2012; Li, 2013). In line with this, we observed an increased senescence-associated phenotype and reduced CD11b immunoreactivity when the mildly activated cells were exposed to Aβ, corroborating findings that have shown increased senescence of astrocytes with aging and AD (Bhat et al., 2012), as well as the observation of dystrophic microglia in the brain of AD patients (Streit et al., 2009). Overall, these results suggest that Aβ treatment impair the ability of young microglia to carry out its neuroprotective role, although developing an inflammatory response to the stress-stimulating Aβ. All these responses are less noticeable in the aged microglia, which still reacts to Aβ with elevated activity of MMPs, autophagy and CD86 positive cells, accompanied by increased iNOS, TGF-β and TNF-α gene expression. Our results indicate, that neuroinflammation by aged microglia is lower when compared with that of the young cells, which again leads to question the relevance of using NSAIDs in later stages of the disease. However, further studies are needed to confirm the existence of distinct inflammation states during the course of AD. In fact, our data show that young microglia show a predominant M1 phenotype, while aged cells present a mixture of M1 and M2 subtypes, which have been related to ageing and AD (Bachstetter et al., 2015; Grabert et al., 2016).

Overall, our data of **Chapter 3** indicate age-associated behavioral changes which are accentuated by Aβ presence promoting heterogeneous responses in microglia cells during AD onset and progression, suggesting that different therapeutic approaches according to the stage of disease may be required.

Lastly, in our final experimental approach (**Chapter 4**) we determined the temporal expression of microglia specific markers in the 3xTg-AD mice model. This model, in contrast to other familial AD models, including the APP/PS1δE9 and the 5xFAD that do not exhibit the development of neurofibrillary tangles, as well as massive neuronal loss (Schwab et al., 2009), progressively develops both Aβ plaques and neurofibrillary tangles with a temporal and spatial distribution that recapitulates the disease in humans (Oddo et al., 2003a; Oddo et al., 2003b). Here, we show a reduced CD11b expression at the early-stage of the disease (3-months), probably to counteract an inflammatory response to the initial increase of intraneuronal Aβ expression already observed at this time-point. This is in line with the reported decrease of F4/80-positive microglia in 3xTg-AD animals

(Janelsins et al., 2005). In addition, we evaluated microglia inflammatory response and observed a marked decreased of inflammatory molecules, such as TNF-α, IL-1β, IL-6, IL-18, and HMGB1 in the very early stages of disease. However, TNF-α, IL-1β and HMGB1 expression increased at 9-months. As suggested by other studies the depressed microglia pro-inflammatory phenotype in an early-stage of disease switch to M1 polarized microglia (Landel et al., 2014; Martin et al., 2017). In addition, our data show that both M1 (MHC class II and CEBP-α) and M2 (SOCS1, TGF-β and Arginase 1) phenotypes were decreased in our mice model in early stages when cortical intraneuronal Aβ appears, increasing at 9-months only the ones related with an innate immune response (iNOS) and a specific deactivated polarization (SOCS1 and Arginase 1). Inflamma-miRNAs, such as miR-155 and miR-146a, were shown to be increased in AD patient brains (Alexandrov et al., 2012) while miR-124 was reduced (Wang et al., 2011). Here, we observed that all the inflamma-miRNA were increased at the early stage of the disease, except miR-155 that was increased and with a sustained elevation at a late stage. Furthermore, since miR-155 is considered a pro-inflammatory marker with early appearance relatively to other inflammatory markers, this fact suggests that it may be an early biomarker for the onset of AD. In addition, miR-155 target analysis showed a predominant downregulation in the 3xTg-AD animals by comparison with the wild type mice, both at 3-months and 9-months of age.

Altogether, these data indicate an immunodepressed state of microglia at early-AD stage in the 3xTg-AD mice model, probably as a reaction to the presence of intraneuronal Aβ, probably as a defense mechanism, that is followed by an upregulation of some inflammatory biomarkers when extracellular Aβ deposition is detected. Further, miR-155 revealed to be an early biomarker of inflammation in AD which may be targeting important microglial immune functions.

Altogether, the results presented in this thesis are schematically represented in **Figure 5.1**. Here, we developed an *in vitro* experimental model to age microglial cells which showed to become dysfunctional and may justify the association of age with AD symptoms. Moreover, treatment of microglia with Aβ further clarified the distinct reactivity of young/activated and aged microglia to a stressing stimulus, which corroborates the additional microglia dysfunction and disease progression upon Aβ accumulation. On the other hand, the *in vivo* studies revealed that the presence of intraneuronal Aβ may lead to an immunodepressed microglial response at early-AD stage, while the later accumulation of extracellular Aβ upregulated some inflammatory biomarkers, being miR-155 an early biomarker of inflammation in AD. Overall, these findings show different

microglia reactivity during disease onset and progression, reinforcing the need to have different therapeutic approaches depending on the stage of AD.

To note that given the high failure rate of clinical trials of AD therapeutics (>99%) after revealing clear efficacy in preclinical trials using these animal models (Cummings et al., 2014), indicates an urgent need of other experimental models that better recapitulate the disease to better understand the differences between early and late microglial inflammatory responses, and validate their modulation by using more directed and specific therapeutic approaches. One of the most attractive, innovative and improved model are the promising use of neural cells and microglia derived from iPSCs generated from AD patients. Such human microglia, better recapitulating the cellular response of the AD patients may be represent a substantial advance to identify microglia subtypes, biomarkers for early AD diagnosis and molecular targets in a human context, leading to the design of new therapeutic strategies and medicines to efficiently prevent and control disease progression.

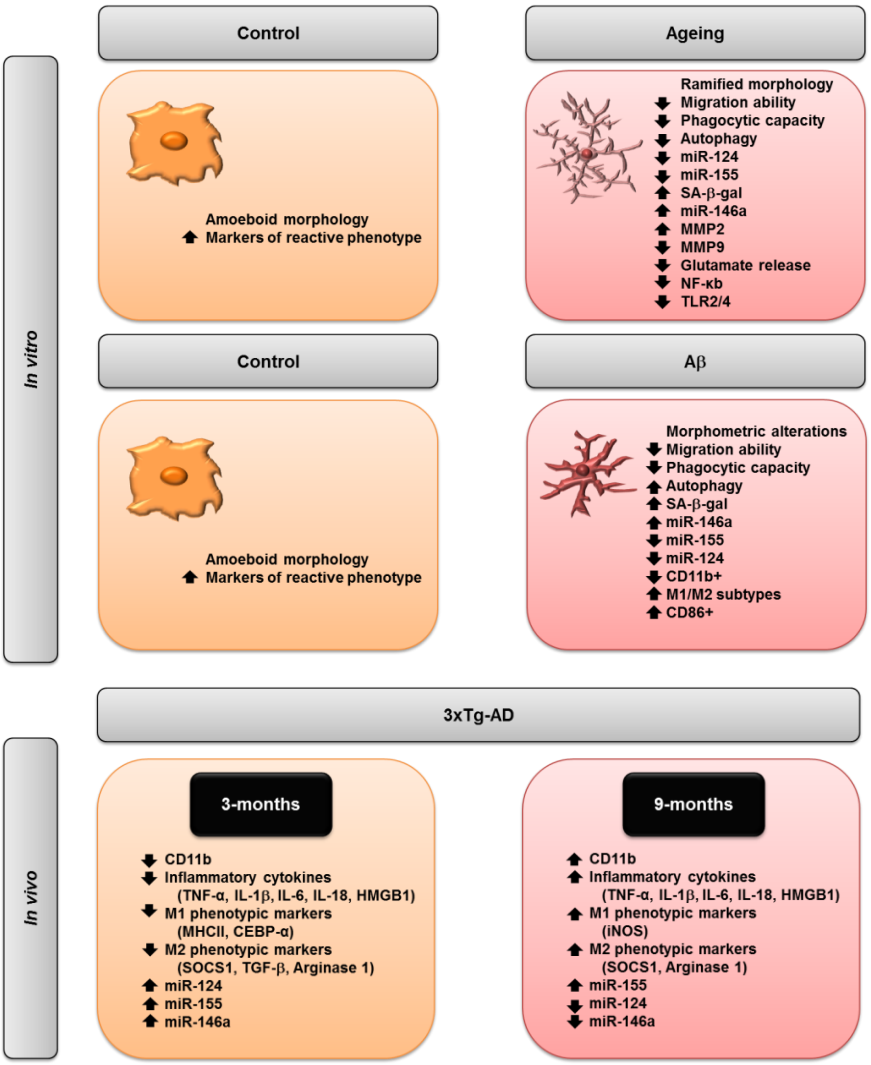

Figure 5. 1 - Schematic representation of the major findings achieved in the present work. Studies performed *in vitro* in microglia primary cultures from neonatal CD1 mice, showed that ageing switches microglia into a more age-like/desensitized phenotype, characterized by a ramified morphology, impaired migration, reduced phagocytic and autophagic ability, decreased expression of inflamma-microRNAs (miR-155 and miR-124) and increased senescence [senescence-associated β-galactosidase (SA-β-gal) and miR-146a]. It was also observed increased matrix metalloproteinase (MMP)-2 activity, accompanied by decreased MMP9 and glutamate release, as well as reduced nuclear factor-kappaB (NF-κB) and Toll-like receptors (TLR)-2 and TLR4. In addition, treatment with amyloid-β ($A\beta_{1-42}$) peptide induced a mixture of microglia subtypes, with a predominant deregulated phagocytic and migration abilities, as well as a decreased expression of inflammatory mediators and surface receptors, while promoted autophagy in young cells, and increased the activity of SA-β-gal and of miR-146a expression. Additionally, Aβ promoted a downregulation of miR-155, miR-124 and CD11b reactive cells in young microglia, while increased the number of CD86 positive cells in the aged cell. Finally, *in vivo* studies in 3xTg-AD animal model showed that these mice have a marked reduction of CD11b reactive cells at 3-months, as well as diminished expression of pro-inflammatory cytokines and inflammatory molecules, being some of those elevated at 9-months. M1 pro-inflammatory microglia markers were reduced at 3-months and maintained during disease progression, with the exception of inducible nitric oxide synthase (iNOS), as well as of M2 anti-inflammatory/damage resolution markers. Inflamma-miRNAs showed to be increased at the early-AD stage, and only miR-155 kept this upregulation for the late stage.

2. References

Alexandrov, P.N., Dua, P., Hill, J.M., Bhattacharjee, S., Zhao, Y., and Lukiw, W.J. (2012). microRNA (miRNA) speciation in Alzheimer's disease (AD) cerebrospinal fluid (CSF) and extracellular fluid (ECF). Int J Biochem Mol Biol 3:365-373.

Ardestani, P.M., Evans, A.K., Yi, B., Nguyen, T., Coutellier, L., and Shamloo, M. (2017). Modulation of neuroinflammation and pathology in the 5XFAD mouse model of Alzheimer's disease using a biased and selective beta-1 adrenergic receptor partial agonist. Neuropharmacology 116:371-386. doi:S0028-3908(17)30010-2 [pii]

Bachstetter, A.D., Van Eldik, L.J., Schmitt, F.A., Neltner, J.H., Ighodaro, E.T., Webster, S.J., et al. (2015). Disease-related microglia heterogeneity in the hippocampus of Alzheimer's disease, dementia with Lewy bodies, and hippocampal sclerosis of aging. Acta Neuropathol Commun 3:32. doi:10.1186/s40478-015-0209-z

Bergamini, E. (2006). Autophagy: a cell repair mechanism that retards ageing and age-associated diseases and can be intensified pharmacologically. Mol Aspects Med 27:403-410. doi:S0098-2997(06)00016-1 [pii]

Bhat, R., Crowe, E.P., Bitto, A., Moh, M., Katsetos, C.D., Garcia, F.U., et al. (2012). Astrocyte senescence as a component of Alzheimer's disease. PLoS One 7:e45069. doi:10.1371/journal.pone.0045069

Bonnema, D.D., Webb, C.S., Pennington, W.R., Stroud, R.E., Leonardi, A.E., Clark, L.L., et al. (2007). Effects of age on plasma matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs). J Card Fail 13:530-540. doi:S1071-9164(07)00154-6 [pii]

Cummings, J.L., Morstorf, T., and Zhong, K. (2014). Alzheimer's disease drug-development pipeline: few candidates, frequent failures. Alzheimers Res Ther 6:37. doi:10.1186/alzrt269

Cunningham, C., Campion, S., Lunnon, K., Murray, C.L., Woods, J.F., Deacon, R.M., et al. (2009). Systemic inflammation induces acute behavioral and cognitive changes and accelerates neurodegenerative disease. Biol Psychiatry 65:304-312. doi:S0006-3223(08)00895-0 [pii]

Damani, M.R., Zhao, L., Fontainhas, A.M., Amaral, J., Fariss, R.N., and Wong, W.T. (2011). Age-related alterations in the dynamic behavior of microglia. Aging Cell 10:263-276. doi:10.1111/j.1474-9726.2010.00660.x

Etminan, M., Gill, S., and Samii, A. (2003). Effect of non-steroidal anti-inflammatory drugs on risk of Alzheimer's disease: systematic review and meta-analysis of observational studies. BMJ 327:128. doi:10.1136/bmj.327.7407.128

Falcão, A.S., Fernandes, A., Brito, M.A., Silva, R.F., and Brites, D. (2005). Bilirubin-induced inflammatory response, glutamate release, and cell death in rat cortical astrocytes are enhanced in younger cells. Neurobiol Dis 20:199-206. doi:S0969-9961(05)00077-X [pii]

Falcão, A.S., Fernandes, A., Brito, M.A., Silva, R.F., and Brites, D. (2006). Bilirubin-induced immunostimulant effects and toxicity vary with neural cell type and maturation state. Acta Neuropathol 112:95-105. doi:10.1007/s00401-006-0078-4

Fichtlscherer, S., De Rosa, S., Fox, H., Schwietz, T., Fischer, A., Liebetrau, C., et al. (2010). Circulating microRNAs in patients with coronary artery disease. Circ Res 107:677-684. doi:CIRCRESAHA.109.215566 [pii]

Floden, A.M., and Combs, C.K. (2007). Microglia repetitively isolated from in vitro mixed glial cultures retain their initial phenotype. J Neurosci Methods 164:218-224. doi:S0165-0270(07)00207-5 [pii]

Gingras, M., Gagnon, V., Minotti, S., Durham, H.D., and Berthod, F. (2007). Optimized protocols for isolation of primary motor neurons, astrocytes and microglia from embryonic mouse spinal cord. J Neurosci Methods 163:111-118. doi:S0165-0270(07)00103-3 [pii]

Grabert, K., Michoel, T., Karavolos, M.H., Clohisey, S., Baillie, J.K., Stevens, M.P., et al. (2016). Microglial brain region-dependent diversity and selective regional sensitivities to aging. Nat Neurosci 19:504-516. doi:nn.4222 [pii]

Graeber, M.B., and Streit, W.J. (2010). Microglia: biology and pathology. Acta Neuropathol 119:89-105. doi:10.1007/s00401-009-0622-0

Harry, G.J. (2013). Microglia during development and aging. Pharmacol Ther 139:313-326. doi:10.1016/j.pharmthera.2013.04.013

Helenius, M., Hanninen, M., Lehtinen, S.K., and Salminen, A. (1996). Changes associated with aging and replicative senescence in the regulation of transcription factor nuclear factor-kappa B. Biochem J 318 (Pt 2):603-608.

Janelsins, M.C., Mastrangelo, M.A., Oddo, S., Laferla, F.M., Federoff, H.J., and Bowers, W.J. (2005). Early correlation of microglial activation with enhanced tumor necrosis factor-alpha and monocyte chemoattractant protein-1 expression specifically within the entorhinal cortex of triple transgenic Alzheimer's disease mice. J Neuroinflammation 2:23. doi:1742-2094-2-23 [pii]

Jiang, M., Xiang, Y., Wang, D., Gao, J., Liu, D., Liu, Y., et al. (2012). Dysregulated expression of miR-146a contributes to age-related dysfunction of macrophages. Aging Cell 11:29-40. doi:10.1111/j.1474-9726.2011.00757.x

Kim, H.J., Ajit, D., Peterson, T.S., Wang, Y., Camden, J.M., Gibson Wood, W., et al. (2012). Nucleotides released from Abeta(1)(-)(4)(2) -treated microglial cells increase cell migration and Abeta(1)(-)(4)(2) uptake through P2Y(2) receptor activation. J Neurochem 121:228-238. doi:10.1111/j.1471-4159.2012.07700.x

Krabbe, G., Halle, A., Matyash, V., Rinnenthal, J.L., Eom, G.D., Bernhardt, U., et al. (2013). Functional impairment of microglia coincides with Beta-amyloid deposition in mice with Alzheimer-like pathology. PLoS One 8:e60921. doi:10.1371/journal.pone.0060921

Lai, A.Y., Dibal, C.D., Armitage, G.A., Winship, I.R., and Todd, K.G. (2013). Distinct activation profiles in microglia of different ages: a systematic study in isolated embryonic to aged microglial cultures. Neuroscience 254:185-195. doi:S0306-4522(13)00781-1 [pii]

Landel, V., Baranger, K., Virard, I., Loriod, B., Khrestchatisky, M., Rivera, S., et al. (2014). Temporal gene profiling of the 5XFAD transgenic mouse model highlights the importance of microglial activation in Alzheimer's disease. Mol Neurodegener 9:33. doi:#N/A [pii]

Li, W. (2012). Eat-me signals: keys to molecular phagocyte biology and "appetite" control. J Cell Physiol 227:1291-1297. doi:10.1002/jcp.22815

Li, W. (2013). Phagocyte dysfunction, tissue aging and degeneration. Ageing Res Rev 12:1005-1012. doi:10.1016/j.arr.2013.05.006

Martin, B.K., Szekely, C., Brandt, J., Piantadosi, S., Breitner, J.C., Craft, S., et al. (2008). Cognitive function over time in the Alzheimer's Disease Anti-inflammatory Prevention Trial (ADAPT): results of a randomized, controlled trial of naproxen and celecoxib. Arch Neurol 65:896-905. doi:2008.65.7.nct70006 [pii]

Martin, E., Boucher, C., Fontaine, B., and Delarasse, C. (2017). Distinct inflammatory phenotypes of microglia and monocyte-derived macrophages in Alzheimer's disease models: effects of aging and amyloid pathology. Aging Cell 16:27-38. doi:10.1111/acel.12522

Minkeviciene, R., Ihalainen, J., Malm, T., Matilainen, O., Keksa-Goldsteine, V., Goldsteins, G., et al. (2008). Age-related decrease in stimulated glutamate release and vesicular glutamate transporters in APP/PS1 transgenic and wild-type mice. J Neurochem 105:584-594. doi:JNC5147 [pii]

Napoli, I., and Neumann, H. (2009). Microglial clearance function in health and disease. Neuroscience 158:1030-1038. doi:S0306-4522(08)00972-X [pii]

Neumann, H., Kotter, M.R., and Franklin, R.J. (2009). Debris clearance by microglia: an essential link between degeneration and regeneration. Brain 132:288-295. doi:awn109 [pii]

Njie, E.G., Boelen, E., Stassen, F.R., Steinbusch, H.W., Borchelt, D.R., and Streit, W.J. (2012). Ex vivo cultures of microglia from young and aged rodent brain reveal age-related changes in microglial function. Neurobiol Aging 33:195 e191-112. doi:10.1016/j.neurobiolaging.2010.05.008

Noren Hooten, N., Abdelmohsen, K., Gorospe, M., Ejiogu, N., Zonderman, A.B., and Evans, M.K. (2010). microRNA expression patterns reveal differential expression of target genes with age. PLoS One 5:e10724. doi:10.1371/journal.pone.0010724

Oddo, S., Caccamo, A., Kitazawa, M., Tseng, B.P., and Laferla, F.M. (2003a). Amyloid deposition precedes tangle formation in a triple transgenic model of Alzheimer's disease. Neurobiol Aging 24:1063-1070. doi:S0197458003002033 [pii]

Oddo, S., Caccamo, A., Shepherd, J.D., Murphy, M.P., Golde, T.E., Kayed, R., et al. (2003b). Triple-transgenic model of Alzheimer's disease with plaques and tangles: intracellular Abeta and synaptic dysfunction. Neuron 39:409-421. doi:S0896627303004343 [pii]

Paczek, L., Michalska, W., and Bartlomiejczyk, I. (2008). Trypsin, elastase, plasmin and MMP-9 activity in the serum during the human ageing process. Age Ageing 37:318-323. doi:afn039 [pii]

Rezai-Zadeh, K., Gate, D., Gowing, G., and Town, T. (2011). How to get from here to there: macrophage recruitment in Alzheimer's disease. Curr Alzheimer Res 8:156-163. doi:BSP/CAR /0144 [pii]

Schwab, C., Klegeris, A., and Mcgeer, P.L. (2009). Inflammation in transgenic mouse models of neurodegenerative disorders. Biochim Biophys Acta 1802:889-902. doi:S0925-4439(09)00249-X [pii]

Sierra, A., Gottfried-Blackmore, A.C., Mcewen, B.S., and Bulloch, K. (2007). Microglia derived from aging mice exhibit an altered inflammatory profile. Glia 55:412-424. doi:10.1002/glia.20468

Smith-Vikos, T., and Slack, F.J. (2012). MicroRNAs and their roles in aging. J Cell Sci 125:7-17. doi:125/1/7 [pii]

Streit, W.J., Braak, H., Xue, Q.S., and Bechmann, I. (2009). Dystrophic (senescent) rather than activated microglial cells are associated with tau pathology and likely precede neurodegeneration in Alzheimer's disease. Acta Neuropathol 118:475-485. doi:10.1007/s00401-009-0556-6

Streit, W.J., Sammons, N.W., Kuhns, A.J., and Sparks, D.L. (2004). Dystrophic microglia in the aging human brain. Glia 45:208-212. doi:10.1002/glia.10319

Streit, W.J., and Xue, Q.S. (2012). Alzheimer's disease, neuroprotection, and CNS immunosenescence. Front Pharmacol 3:138. doi:10.3389/fphar.2012.00138

Von Bernhardi, R. (2010). Immunotherapy in Alzheimer's disease: where do we stand? Where should we go? J Alzheimers Dis 19:405-421. doi:10.3233/JAD-2010-1248

Weggen, S., Eriksen, J.L., Das, P., Sagi, S.A., Wang, R., Pietrzik, C.U., et al. (2001). A subset of NSAIDs lower amyloidogenic Abeta42 independently of cyclooxygenase activity. Nature 414:212-216. doi:10.1038/35102591